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(54) Title: ANTISENSE INHIBITORS OF GENE EXPRESSION

(57) Abstract

This invention is a new synthetic method for the preparation of oligonucleotide analogs containing a neutral 5'-thioformacetal internucleoside linkage and new di- and trinucleotide analogues containing purines and pyrimidines with neutral 5'-thioformacetal internucleoside linkages.

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TITLE OF THE INVENTION

ANTISENSE INHIBITORS OF GENE EXPRESSION

BACKGROUND OF THE INVENTION

1. Field of the Invention:

This patent disclosure is concerned with a new synthetic method for the preparation of oligonucleotide analogs containing a neutral 5'-thioformacetal internucleoside linkage and new di- and trinucleotide analogues containing purines and pyrimidines with neutral 5'-thioformacetal internucleoside linkages. These new compounds may be used as antisense inhibitors of gene expression and as anti-viral or anti-cancer agents.

2. Background of the Invention:

The use of antisense technology has been recently reviewed by Uhlmann and Peyman, [Chem. Rev. 90:543, 1990]. In essence, this technology involves introduction into living cells of oligonucleotides with sequences complementary to nucleic acids (including regulatory elements and structural elements found on DNA or RNA) present in a host cell. The introduced oligonucleotide is able to bind to and thereby interrupt the expression of undesirable gene products within the host cell.

Matteucci et al., [J. Am. Chem. Soc. 113:7767-7768, 1991] reported on deoxynucleotides bearing neutral analogs of the phosphodiester linkage which could recognize duplex DNA via triple helix formation. That paper disclosed a dipyrimidine analog. The method of preparation of Matteucci et al., is inefficient for the preparation of analogs containing purines.

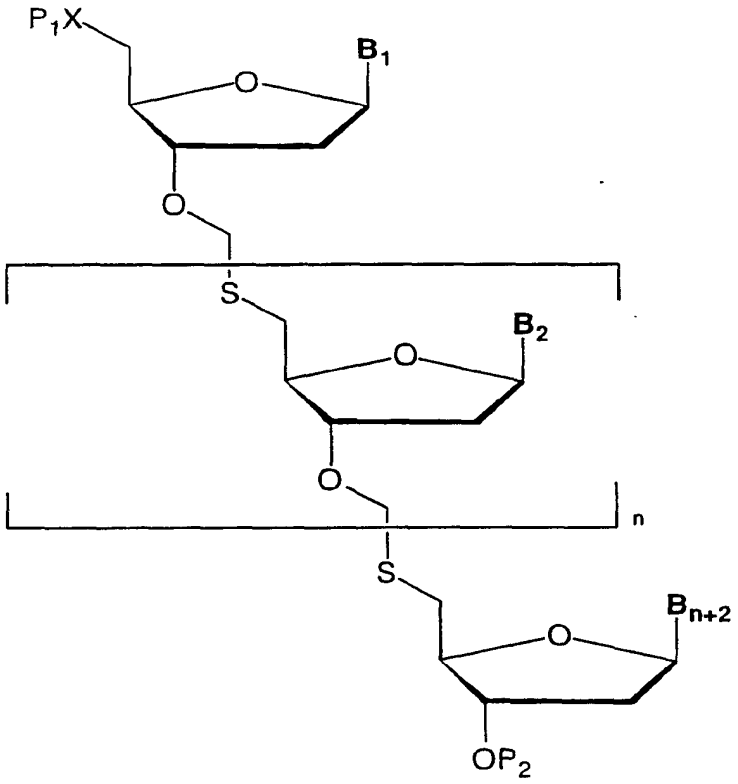
Jones et al., [J. Org. Chem. 58:2983-2991, 1993] disclosed 3'-thioformacetal and formacetal dinucleotide analogs.

Kaway et al., [Can. J. Chem. 70:1573-1580, 1992] disclosed the synthesis of 5'-deoxy-5'-thiothymidine.

Benneche et al., [Acta Chem. Scan. B37:93-96, 1983] and Zavgorodny et al., [Tetrahedron Lett. 51:7593-7596, 1991] disclosed the use of sulfuryl chloride for the preparation of O,S-acetals.

SUMMARY OF THE INVENTION

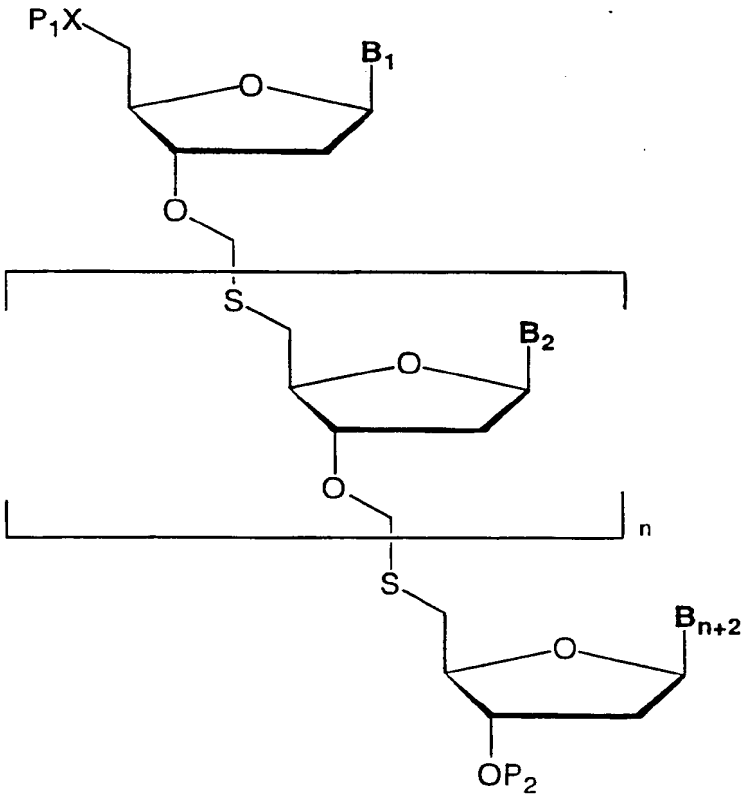
This invention relates to novel compounds and a new synthetic method for the preparation of oligonucleotide analogs containing a neutral 5'-thioformacetal internucleoside linkage and new di-and trinucleotide analogues containing purines and pyrimidines with neutral 5'-thioformacetal internucleoside linkages. Compounds of this invention prepared according to the novel method disclosed herein have the formula:



wherein:
B_1, B_2, and B_{n+2} are naturally occurring or non-naturally occurring nucleic acid bases, including but not limited to purines or pyrimidines selected from adenine, thymine, guanine, cytosine, uracil, and inosine;
P_1 and P_2 are independently H, lower alkyl, acyl, substituted or unsubstituted trityl or trialkylsilyl;
X is O, or S; and
n is a number from 0 to 28.

DETAILED DESCRIPTION OF THE INVENTION

Natural oligonucleotides are easily degraded by intracellular nucleases and do not diffuse efficiently through cell membranes. Replacement of the natural phosphodiester backbone by the uncharged 5'-thioformacetal backbone reduces these problems. Thus, according to our invention there is provided compounds of the general formula:



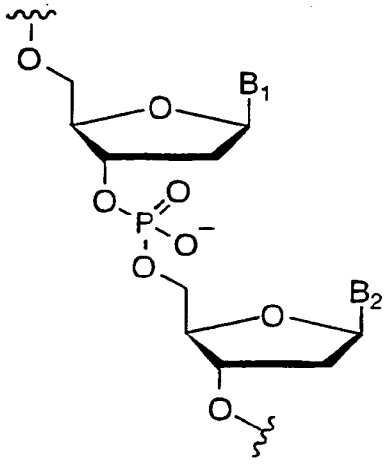
wherein:
B_1, B_2, and B_{n+2} are naturally occurring or non-naturally occurring nucleic acid bases, including but not limited to purines or pyrimidines selected from adenine, thymine, guanine, cytosine, uracil, and inosine;
P_1 and P_2 are independently H, lower alkyl, acyl, substituted or unsubstituted trityl or trialkylsilyl;
X is O, or S; and
n is a number from 0 to 28.

In practicing this invention, it should be borne in mind that nucleic acids depend on molecular recognition for molecular function. This is achieved through the ability of purines and pyrimidines to hydrogen bond with each other. The bases which stably hydrogen bond are referred to as complementary bases. In general, adenine is complementary to thymine and cytosine is complementary to guanine. Inosine bonds to either cytosine, uracil or thymine. In a naturally occuring DNA or RNA molecule, the bases are typically linked to a sugar molecule, usually a pentose, selected from D-ribose (in RNA) or 2-deoxy-D-ribose (in DNA), and is then referred to as a nucleoside. In a nucleoside, the glycosidic C-1 carbon atom of the pentose is bonded to N-1 of the pyrimidine or N-9 of the purine base. The naturally occuring nucleosides are adenosine, guanosine, uridine, cytidine, deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. Finally, each nucleoside is linked to a phosphate moiety to form a nucleotide. In the naturally occurring polymers DNA and RNA, the nucleotides are polymerized through phosphodiester linkages.

For the purposes of anti-sense inhibition of gene expression, the natural linkages between nucleotides is not adequate. The charged nature of the phosphodiester bonds renders oligonucleotides impermeable to cellular membranes. Furthermore, the natural linkage is susceptible to cleavage by cellular enzymes such as phosphodiesterases. By contrast, this invention discloses compounds that have neither of these problems. The differences between the natural linkage and the linkage in the

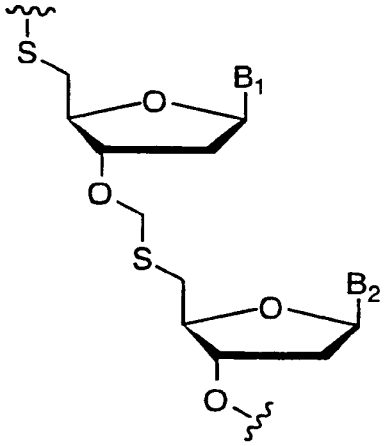
product of this invention is depicted below, along with a notation of several improved features:

DNA



- Ionic

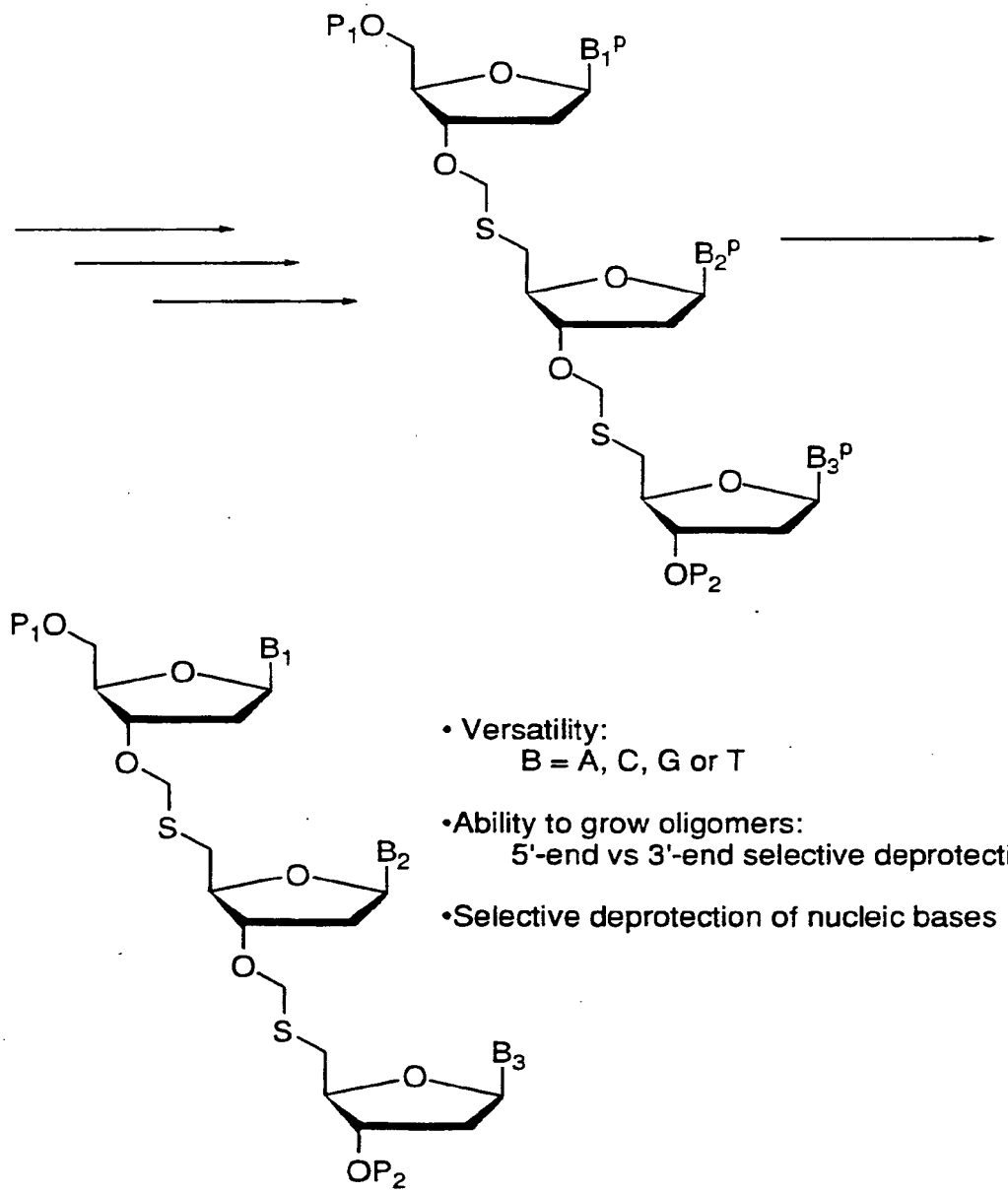
DNA 5'-Thio Formacetal Analogue



- Neutral
- Achiral
- Isosteric to phosphate

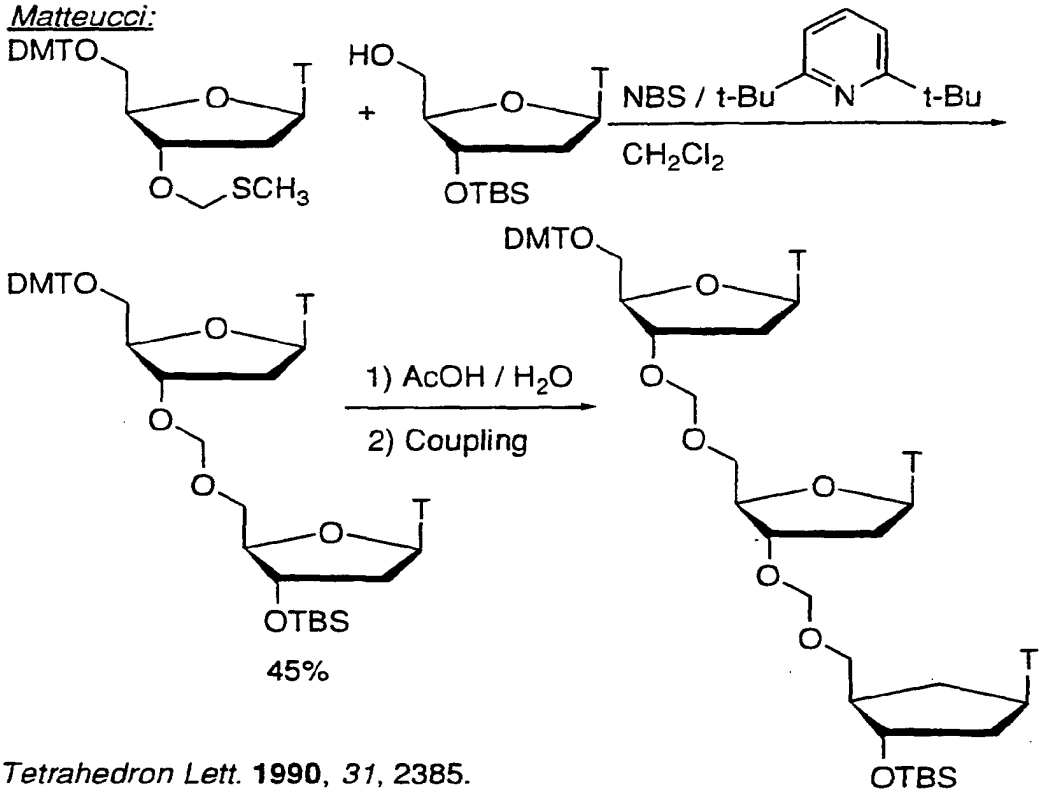
In order to produce the instant compounds, it is necessary that a versatile synthetic method be developed. It is an object of this invention to provide a method which permits production of antisense inhibitors incorporating both purines and pyrimidines. It is also an objective to provide a method which allows extension of the oligomer via either 5'-end or 3'-end deprotection, and further, to provide selective deprotection of nucleic acid bases. The method and advantages are summarized below with reference to synthesis of a trimer:

Synthetic Requirements

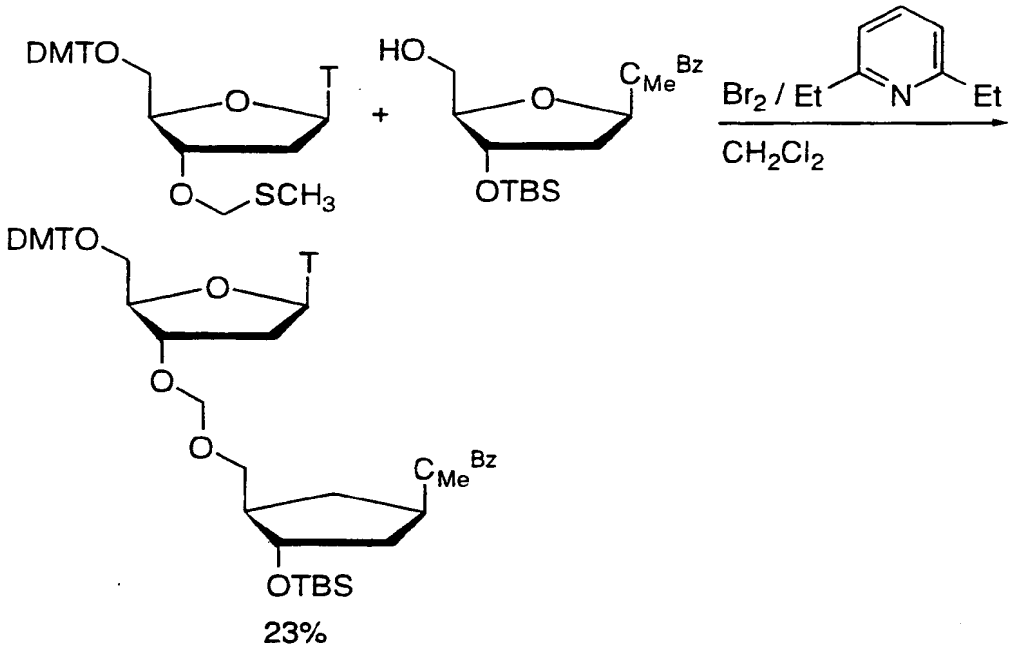


A number of researchers have reported synthetic schemes for producing oligonucleotides with uncharged linkages. However, the chemistry reported has limitations as to the types of bases that may be

incorporated and the types of protection and deprotection available. Reported chemistry is summarized below with an indication of the limits on the known technology:



Tetrahedron Lett. **1990**, *31*, 2385.



J. Org. Chem. **1993**, *58*, 2983.

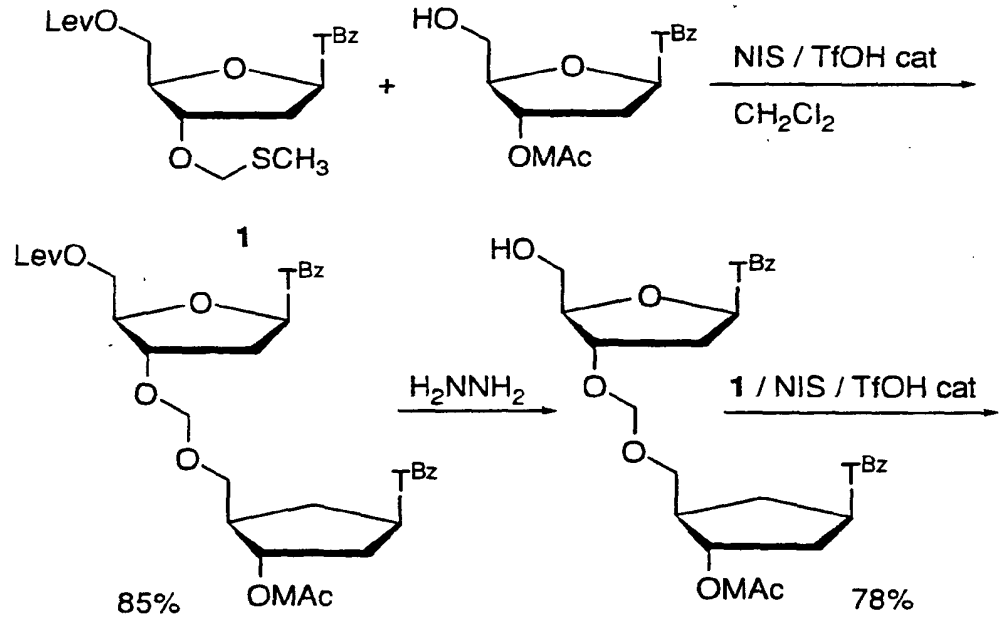
- Ability to grow oligomers: 5'-end vs 3'-end selection deprotection
- Selective deprotection of nucleic bases
- Versatility: only pyrimidines can be coupled.

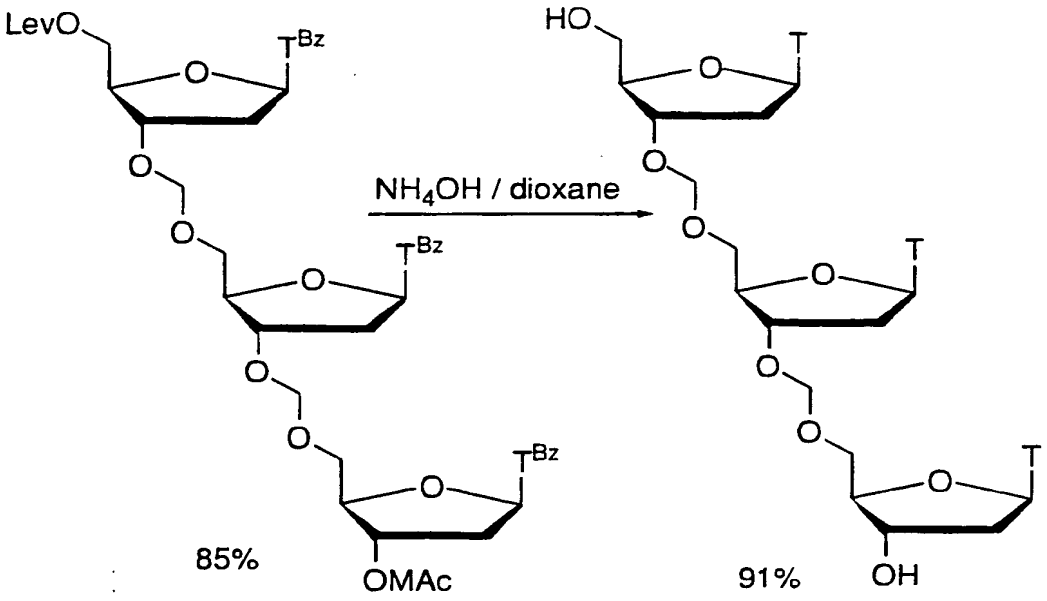
Thus, Matteucci et al., have reported the synthesis of di- and trinucleotide analogs with formacetal linkages by the activation of methylthiomethylacetal donors with N-bromosuccinimide or bromine. This methodology is limited to the coupling of pyrimidine derivatives.

Van Boom et al., have also reported the synthesis of di- and trinucleotide analogs bearing a formacetal linkage by the activation of methylthiomethylacetal donors. Their method relies on the use of N-iodosuccinimide with a a catalytic amount of trifluoromethanesulfonic acid to activate the sulfide moiety and gives access only to thymidine derivatives. The use of the acid dictates the choice of base labile esters as 3' and 5'-OH protecting groups. This makes it impossible to selectively

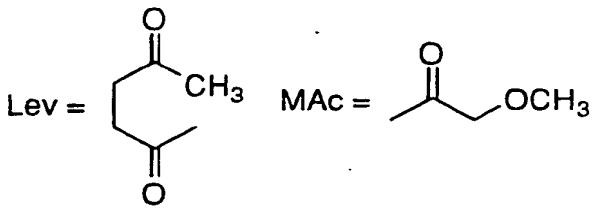
deprotect the nucleic bases. Van Boom et al., subsequently disclosed an alternative approach for the synthesis of formacetal linked dinucleotides. That method uses acetoxymethylacetal donors which are activated by trimethylsilyltrifluoromethanesulfonate (TMSOTf). This method gives access to derivatives of thymidine, deoxycytidine and deoxyguanosine. The missing deoxyadenosine derivatives were obtained later by a variation of the method disclosed by Van Boom et al., whereby (dibutoxyphosphoryloxy)methylacetal donors are activated with TMSOTf. Even with this variation, the ability to synthesize oligonucleotides containing adjacent purines, as in purine-purine dimers, was not reported. Furthermore, the use of a Lewis acid, once again, put constraints on the choice of hydroxyl protecting groups which precludes the selective removal of amide protection on nucleic acid bases. The various methods of Van Boom are summarized below:

Van Boom:



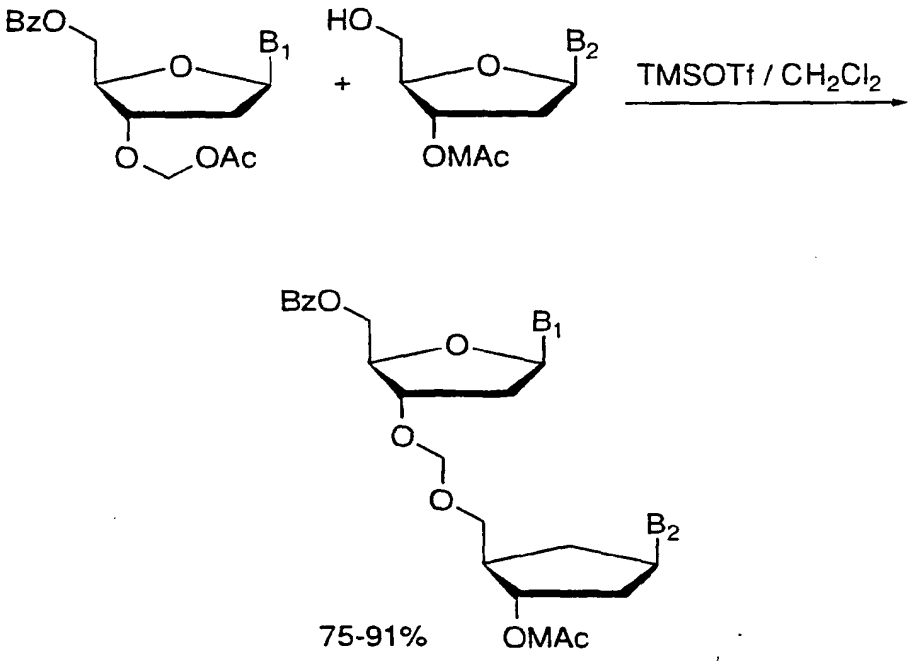


Tetrahedron **1991**, *47*, 1547.



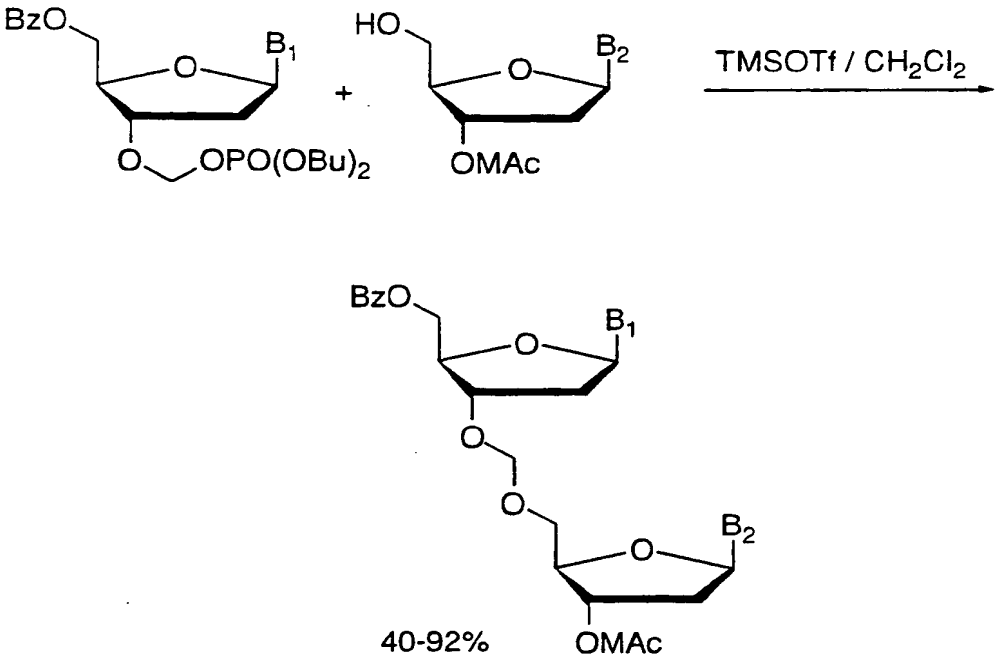
• Ability to grow oligomers.	• Unselective deprotection of nucleic bases. • Versatility: only thymidine can be coupled.

Van Boom:



Tetrahedron Lett. **1992**, *21*, 3081.

• Versatility: B_1 = T, C^{Bz} B_2 = T, C^{Bz}, G^{iBu} ≠ A^{Bz}	• Unselective deprotection of nucleic bases.

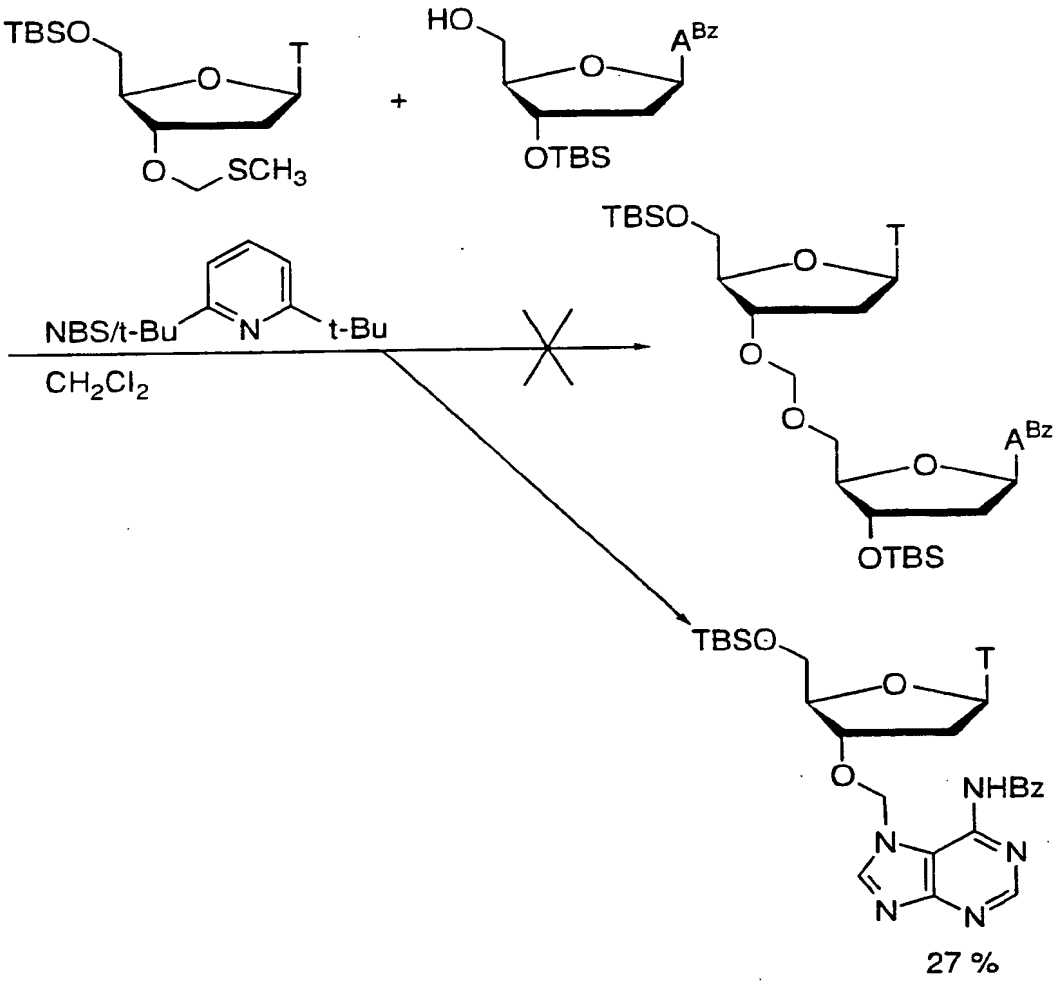


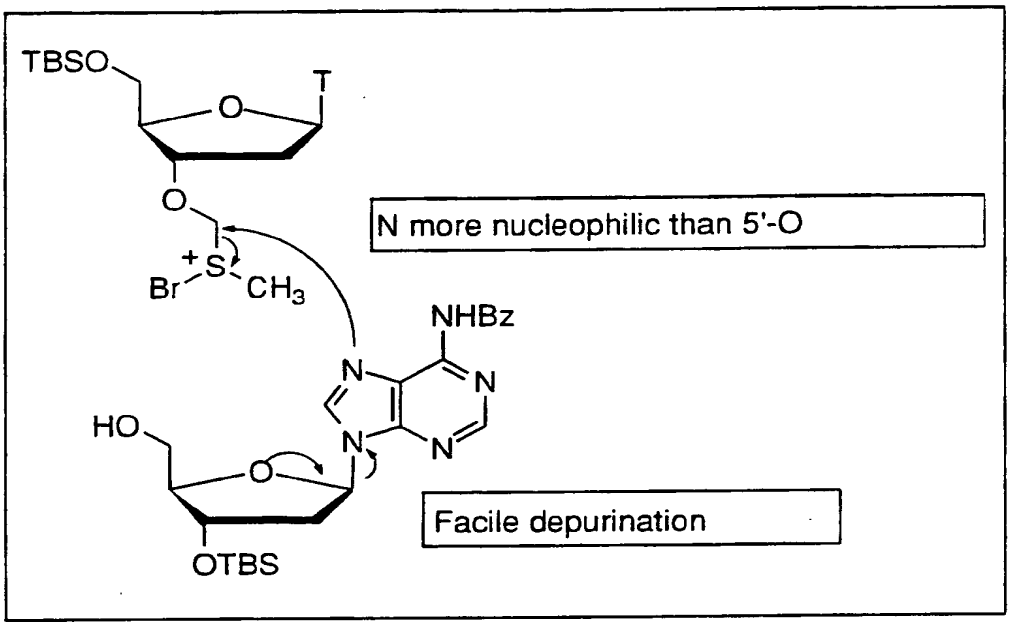
Synthesis **1993**, 3081.

• Ability to grow oligomers.	• Unselective deprotection of nucleic bases.
• Versatility: B_1 = T, C^{Bz}, G^{iBu}, A^{Bz} B_2 = T, C^{Bz}, G^{iBu}, A^{Bz} No Purine-Purine dimers	

From the foregoing discussion, it is clear that there are problems in the art of purine coupling which severly limit the ability to produce relevant antisense inhibitory compounds. These problems are further understood with regard to the following scheme. The scheme shows that in attempting to couple a purine, known methods result in facile depurination due to the more nucleophilic nature of nitrogen than the 5'-oxygen. The following scheme also indicates the solution defined by the instant inventors of replacing the 5'-oxygen with the more nucleophilic sulfur atom.

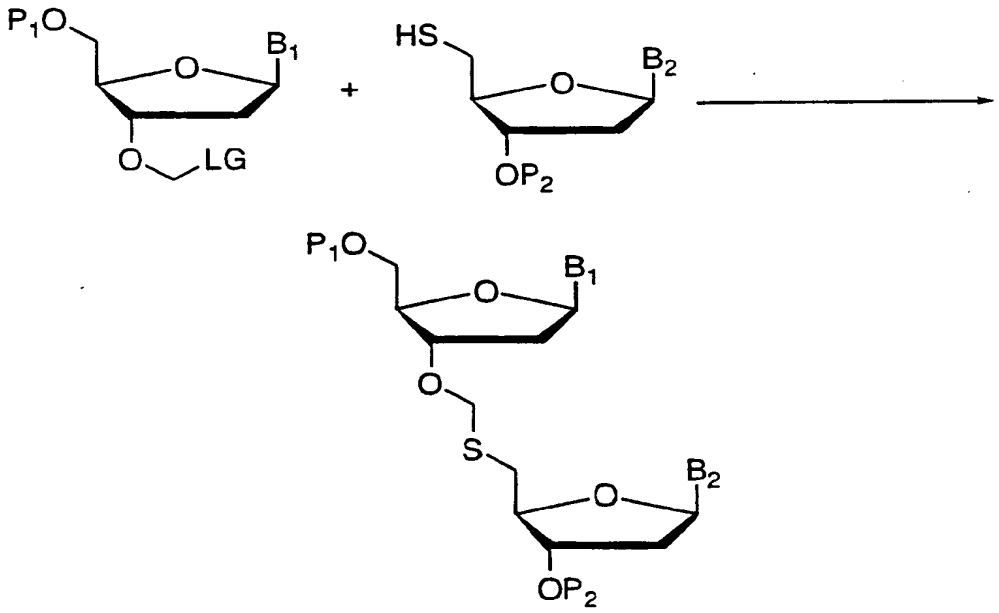
The problem with purine coupling





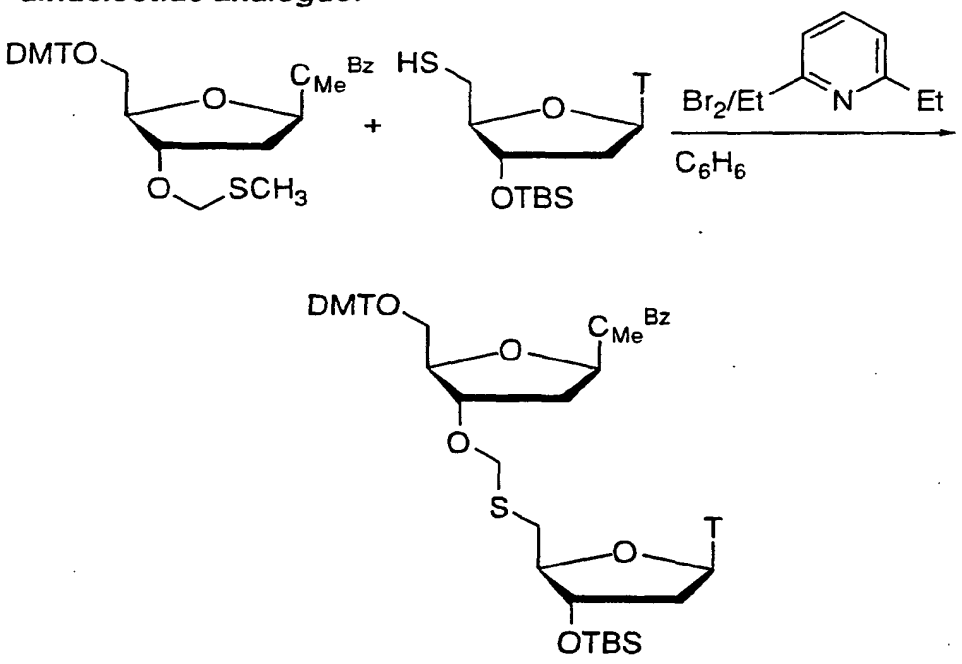
A Solution:

- Replace 5'-oxygene by a more nucleophilic sulfur atom.



In one report, formation of a 5'-thioformacetal dinucleotide was provided without any yield. The applicability of the reported method to solving the aforementioned problems is difficult to ascertain. The proposed method is as follows: Bromine activation of a methylthiomethylacetal donor followed by addition of a 5'-thiothymidine derivative to give a pyrimidine-pyrimidine dinucleotide analog. However, in our hands, this method did not give access to purine analogs. The proposed method is shown below:

Report of a 5'-thioformacetal dinucleotide analogue.

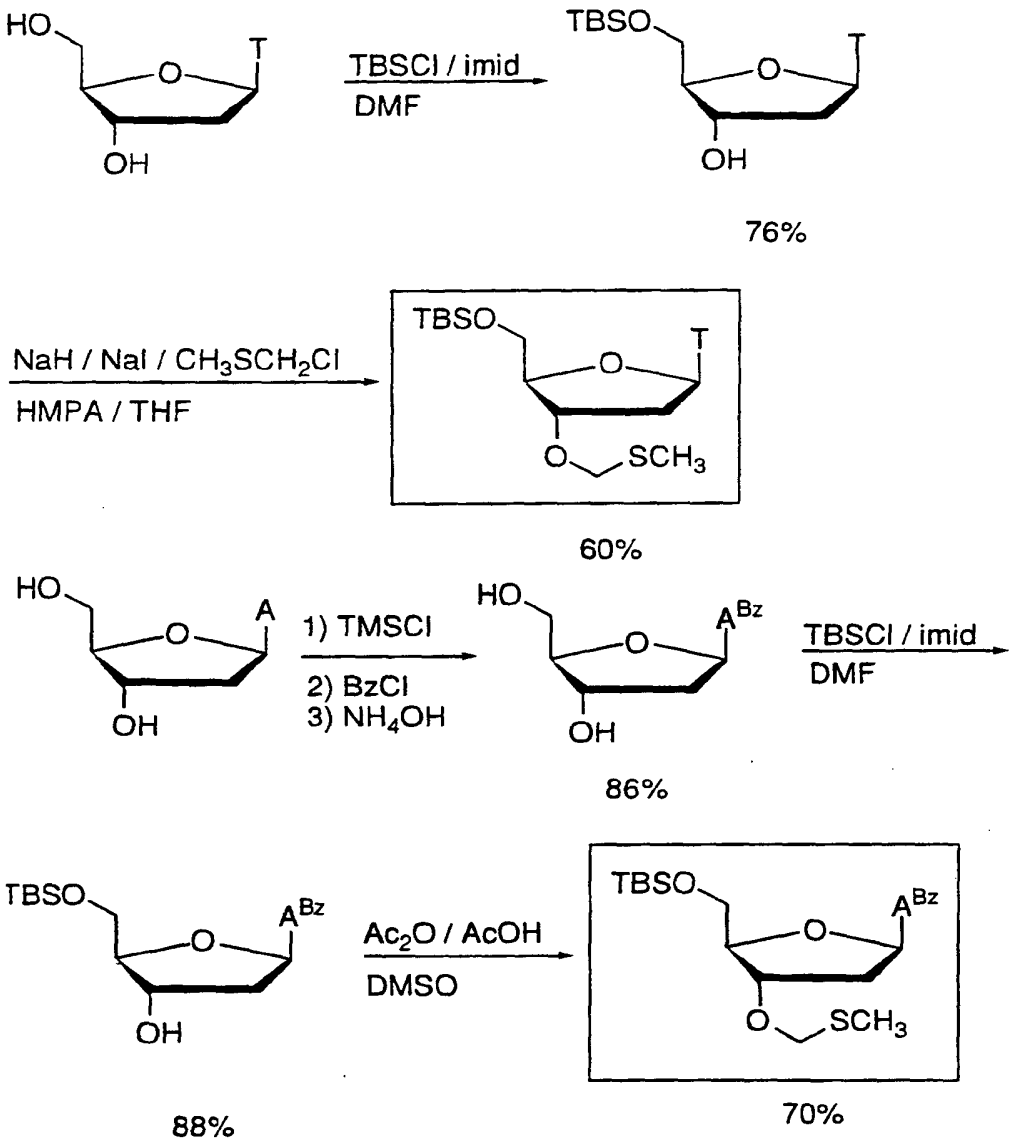


No yield reported

M. Matteucci *et al.*, *J. Am. Chem. Soc.* **1991**, *113*, 7767.

According to the method of the instant invention, a nucleoside donor and a nucleoside acceptor are prepared in good yield as shown in the following two schemes. The preparation of nucleoside donors starts with the selective 5'-O-silylation of suitably base-protected deoxynucleosides. Alkylation of the secondary 3'-hydroxyl group is then accomplished by treatment with chloromethyl methyl sulfide or by a Pummerer reaction [Pojer, P.M. and Angyal, S.J. Tetrahedron Lett. 35:3067, 1976] to afford a 3'-O-methylthiomethylacetal donor. This synthesis is shown in the following scheme:

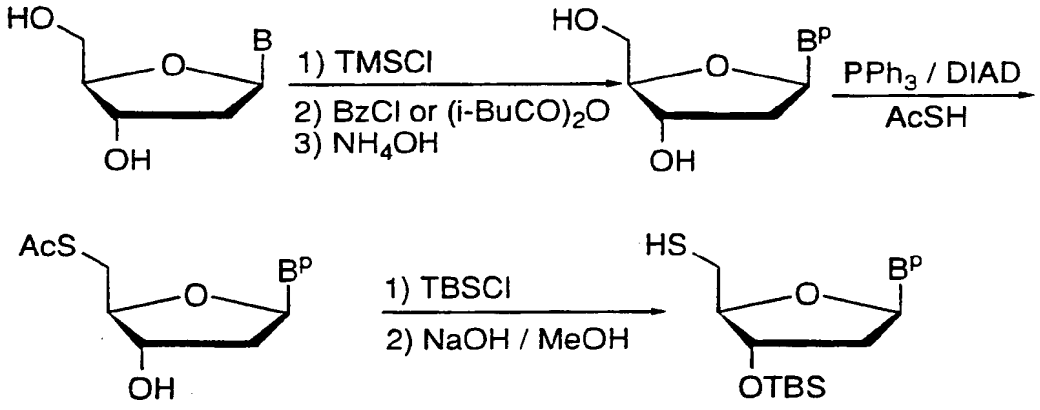
Preparation of Nucleoside Donors:



The first step in the synthesis of nucleoside acceptors involves a Mitsunobu reaction [Mitsunobu, O. Synthesis p.1, 1981] on the primary alcohol of suitably base-protected deoxynucleosides to afford 5'-S-acetyl nucleoside derivatives. Silylation of the secondary alcohol

followed by methanolysis of the thioester provides the desired 5'-thionucleoside derivatives used as nucleoside acceptors. This synthesis is shown in the following scheme:

Preparation of Nucleoside Acceptors:



B	BP	Yields (%)			
		Base Protection	Mitsunobu	Silylation	Methanolysis
T	T	-	42	98	82
C	C^{Bz}	90	41	92	56
G	G^{iBu}	95	90	78	65
A	A^{Bz}	86	77	91	51

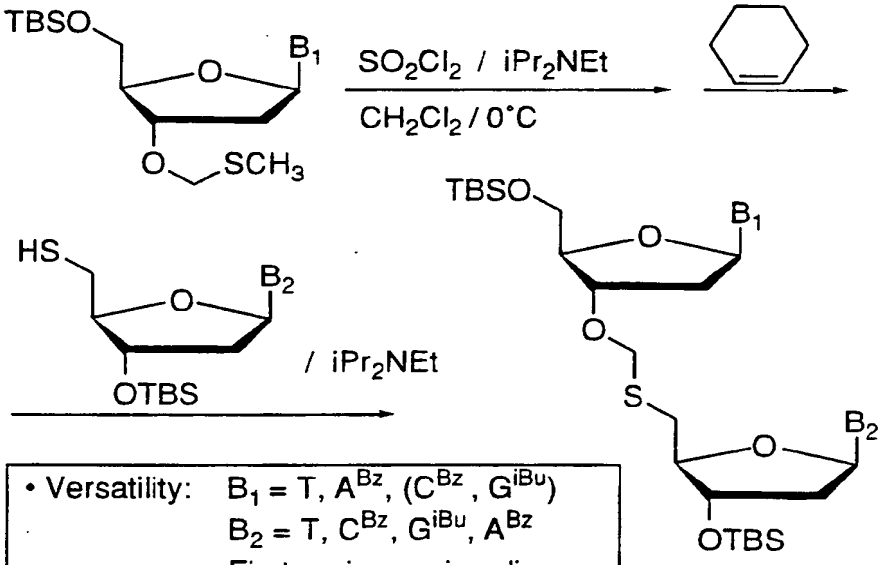
Once the nucleotide donor and acceptor are prepared, the acceptor and donor are coupled as follows:

A mixture of nucleoside donor, N,N-diisopropylamine (about 1.4 equivalents) or a similar reagent and 3 angstrom molecular sieve in dichloromethane or a similar reagent are stirred at about 0°C and then treated with sulfuryl chloride (about 1.3 equivalents). After a short period of about one minute, cyclohexene (about 2 equivalents) is added to trap the methylsulfenyl chloride formed in situ. The reaction mixture is then stirred for an additional short period of about 10 minutes at ambient temperature before a solution of the nucleoside acceptor (about 1.3

equivalents) and N,N-diisopropylamine (about 1.4 equivalents) or a similar reagent in dichloromethane or a similar solvent is added. After allowing the reaction to proceed for several hours (about 3.5 hours is adequate), the volatiles are evaporated and flash chromatography or another separation method is used to fractionate the residue, to afford the 5'-thioformacetal linked dinucleotide or larger analogs.

Accordingly, we report a new method that is versatile, for the formation of purine and pyrimidine containing antisense inhibitors (including adjacent purines, as in purine-purine dimers) of gene expression. The use of sulfuryl chloride to activate the sulfide moiety of nucleoside donors provides a non-acidic method of coupling. As a consequence, purine derivatives as well as pyrimidine derivatives can be used as nucleoside donors. Furthermore, usual acid-labile hydroxyl protecting groups are tolerated, with the result that nucleic acid bases can be selectively deprotected by conventional ammonolysis. This chemistry is summarized in the following scheme:

Formation of 5'-thioformacetal dinucleotides

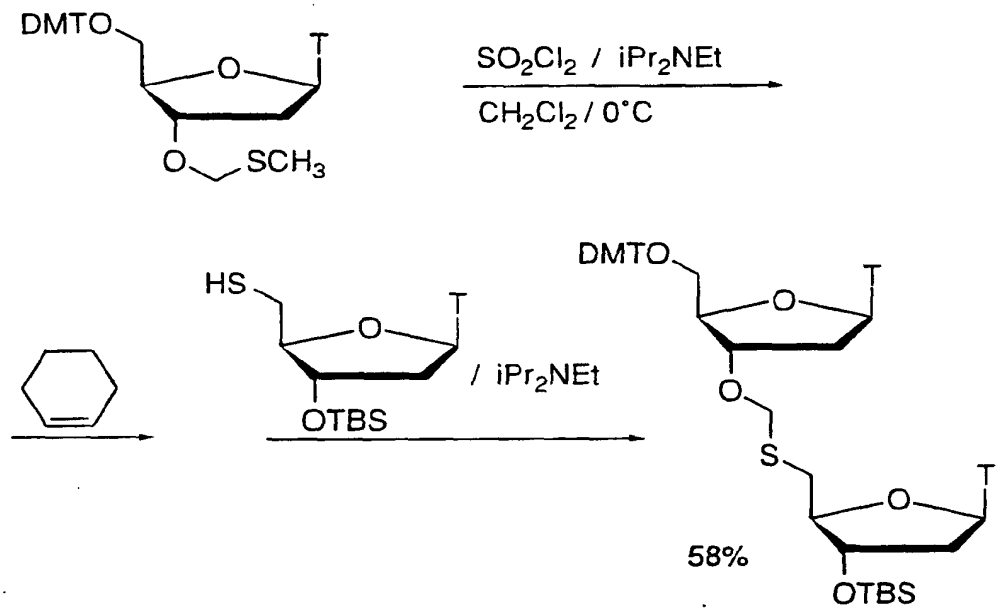


- Versatility: B_1 = T, A^{Bz}, (C^{Bz}, G^{iBu})
  B_2 = T, C^{Bz}, G^{iBu}, A^{Bz}
  First purine-purine dimers reported.

B_1	B_2	Yield (%)
T	T	60
T	C^{Bz}	58
T	G^{iBu}	47
T	A^{Bz}	51
A^{Bz}	T	68
A^{Bz}	C^{Bz}	58
A^{Bz}	G^{iBu}	49
A^{Bz}	A^{Bz}	64

According to the method of this invention, one preferred 5'-end protecting group is dimethoxytrityl, which permits differential 3'-end and 5'-end protection as shown in the following scheme:

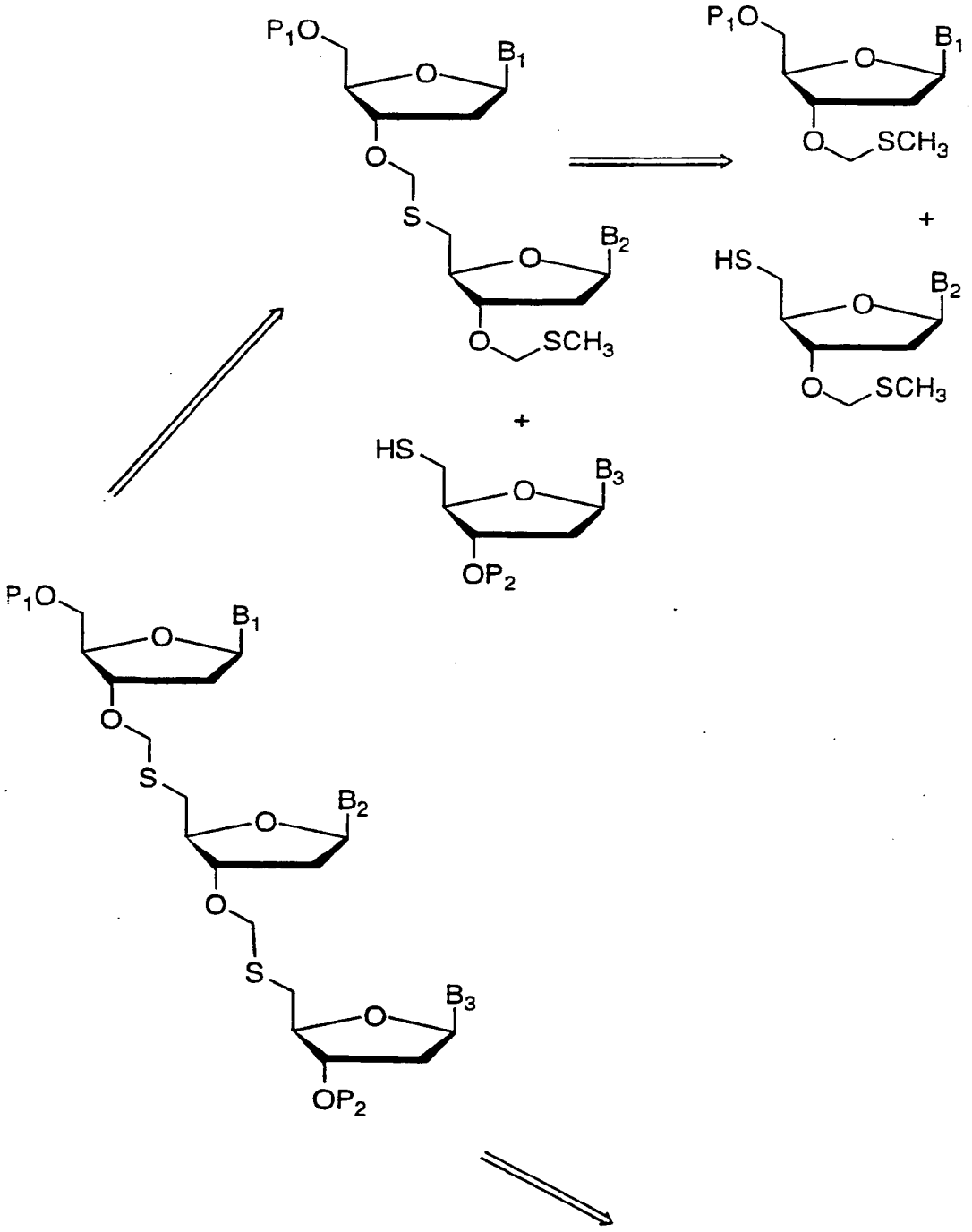
Dimethoxytrityl is a suitable 5'-end protecting group

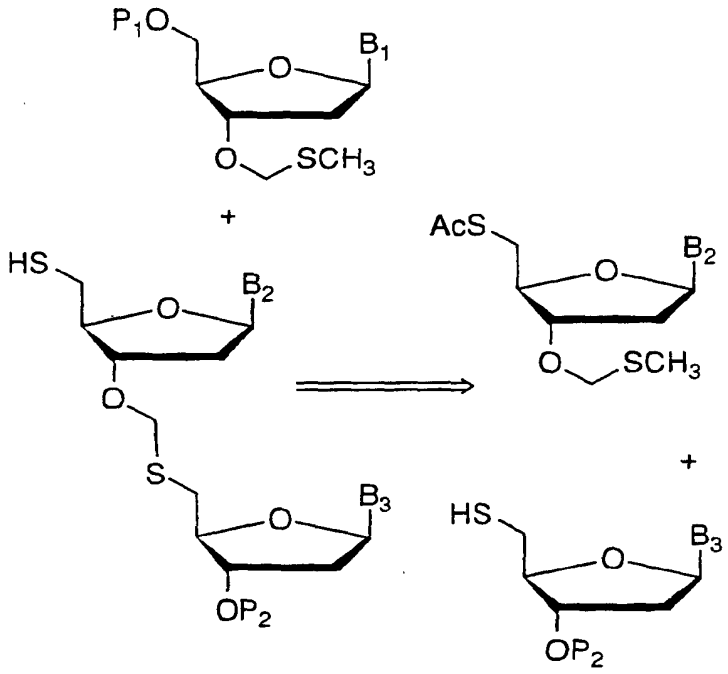


Differential 3' and 5'-end protection

As a result of the novel method of this invention, we have discovered that we can extend the oligomers from either the 3'-end or the 5'-end. As shown in the next scheme, a trinucleotide can be prepared whether by coupling a dinucleotide donor with a nucleoside acceptor or by coupling a nucleoside donor with a dinucleotide acceptor. In like manner, oligonucleotide donors may be prepared up to and including twenty nine-mers, and added to a nucleoside acceptor, or by coupling a nucleoside donor with an oligonucleotide acceptor up to and including twenty nine-mers, to form thirty-mers:

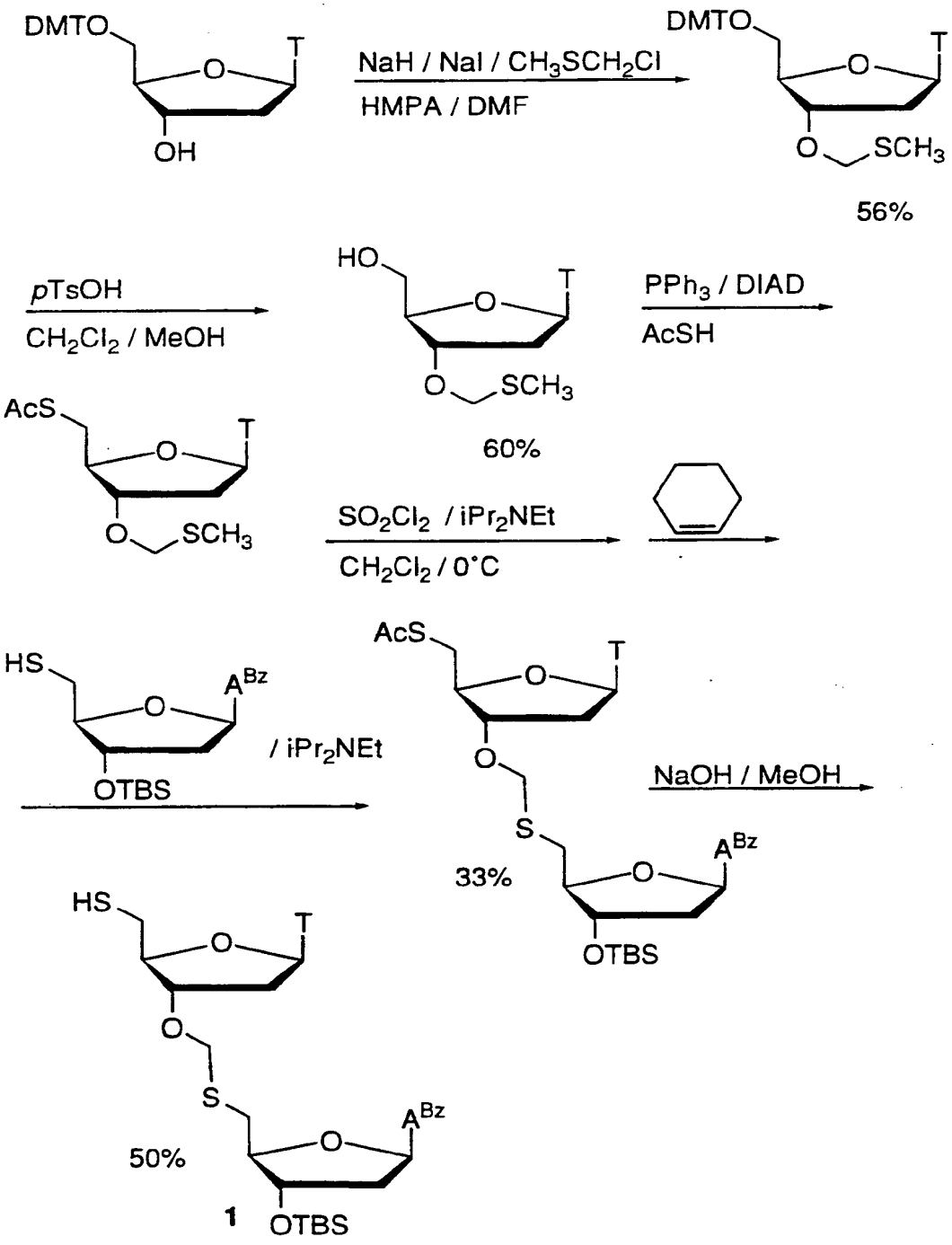
The ability to grow oligomers: Trinucleotide retrosynthesis .

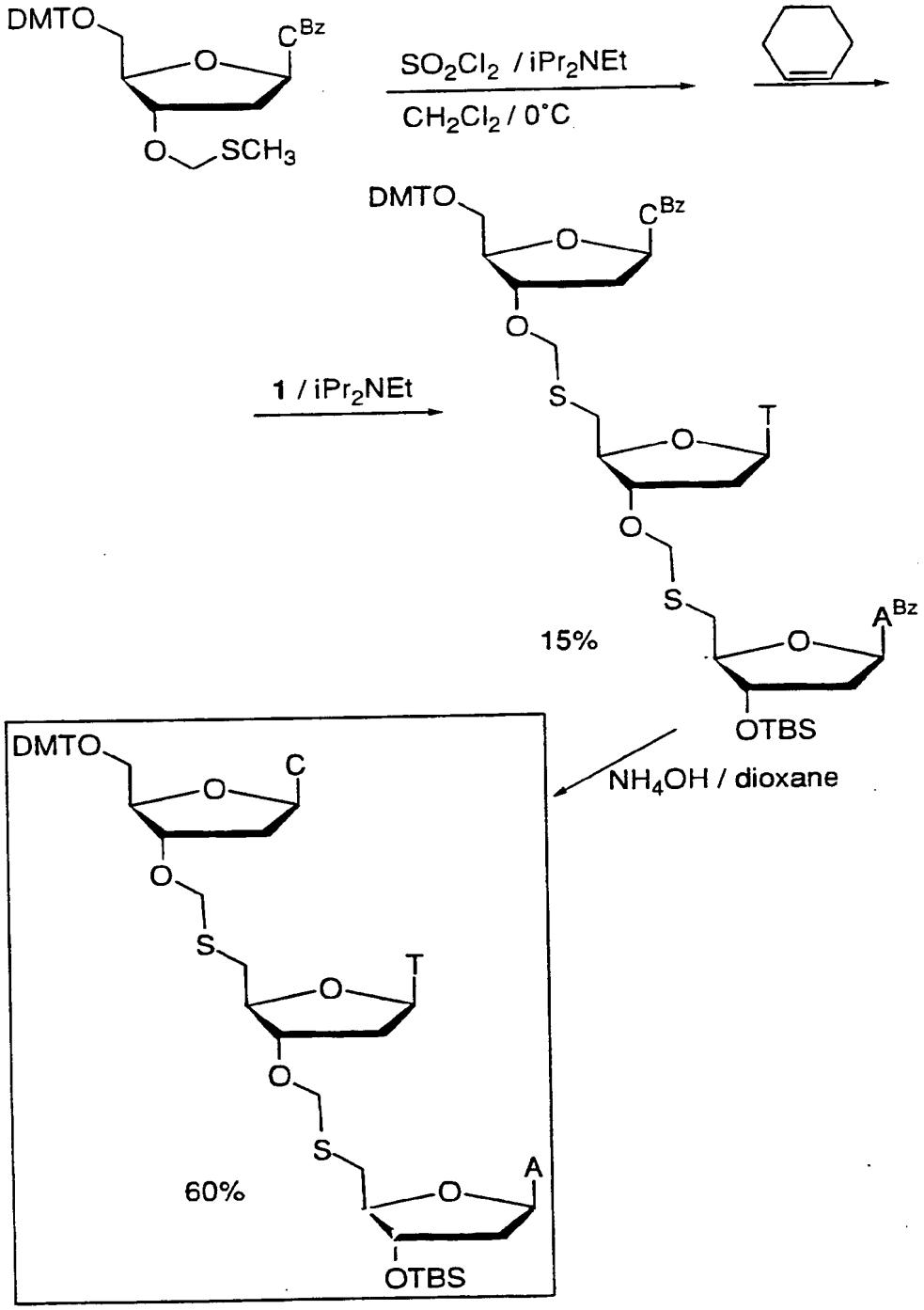




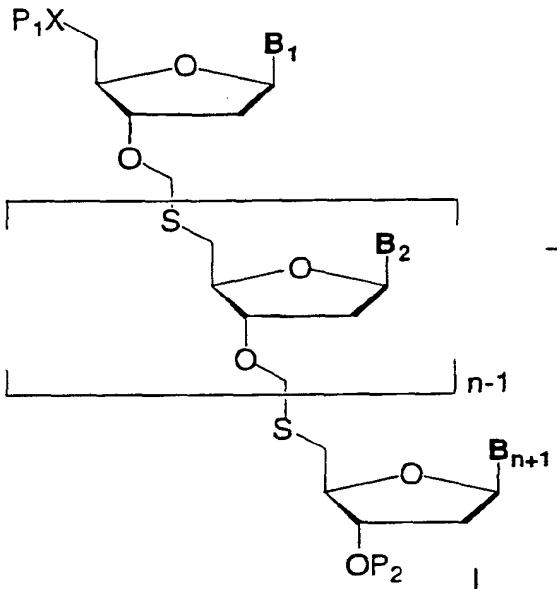
In view of the foregoing discoveries, we report the synthesis of a trinucleotide antisense inhibitor, as shown in the next scheme. The final product shown is a trinucleotide analog with nucleic acid bases unprotected and differentially protected 3' and 5' ends:

The synthesis of a Trinucleotide

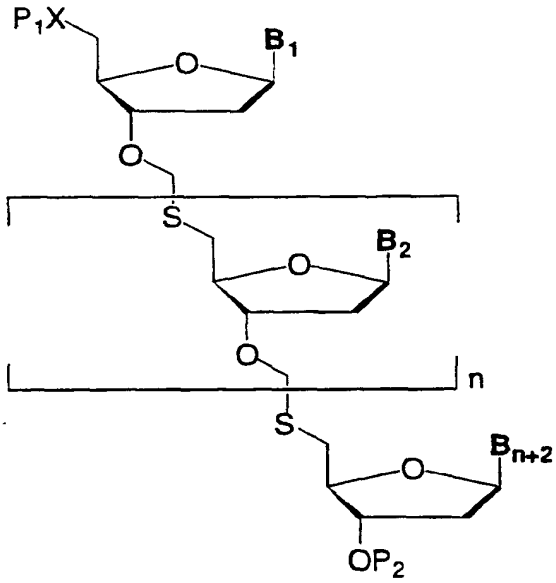




Accordingly, the synthetic methods disclosed above may be generalized to provide oligonucleotide analogs of extended sequence. While no theoretical upper limit to the chemistry disclosed herein is known, practically, for the purposes of anti-sense inhibition of gene expression, oligonucleotides up to about a 30-mer appear adequate [see for example, the discussion in Milligan et al., J. Med. Chem. 36 No.14:1923-1937, 1993, where most of the work done in this field finds success with oligomers shorter than 30-mers. Thus, one embodiment of the invention is a method for preparation of an oligonucleotide analog of formula II from a compund of formula I:



I



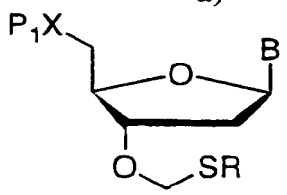
II

wherein:

B_1, B_2, B_{n-1}, B_{n+1} and B_{n+2} are naturally occurring or non-naturally occurring nucleic acid bases;

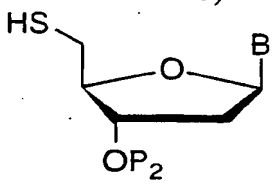
P_1 and P_2 , together are an oligomer up to a length of n, or are independently H, lower alkyl, acyl, substituted or unsubstituted trityl or trialkylsilyl;
X is O, or S; and
n is an number from 0 to 28;
which comprises:

a) preparing a nucleoside donor of formula:



;

b) preparing a nucleoside acceptor of formula:



; and

c) coupling the nucleoside donor and acceptor;

wherein:
B is a naturally occurring or non-naturally occurring nucleic acid purine or a pyrimidine base;
P_1 and P_2 are independently H, lower alkyl, acyl, substituted or unsubstituted trityl or trialkylsilyl;
X is O, or S;
R is a lower alkyl;

d) repeating steps (a)-(c) as many times as required to achieve the compound with the desired sequence having a total of n+2 bases.

The novel nucleoside, dinucleotide, trinucleotide and oligonucleotide analogs of this invention are useful to interrupt expression inside cells of undesirable gene products. The products of this invention may be used as anti-viral or anti-cancer agents. Furthermore, the oligonucleotides of this invention may be incorporated into longer,

oligodeoxynucleotides having naturally occurring phosphodiester backbones, to prepare antisense strands with defined sequences that bind to complementary single-stranded intracellular nucleic acid targets. Such specific interactions inhibit the expression of the genetic information contained in the bound sense strands.

Natural oligonucleotides are easily degraded by intracellular nucleases and do not diffuse efficiently through cell membranes. Replacement of the natural phosphodiester backbone by the uncharged 5'-thioformacetal backbone reduces these problems.

According to the instant invention, any nucleotide sequence encoding an undesirable gene product may be used to prepare an anti-sense inhibitor with a complementary base sequence. Thus, sequences complimentary to sequences encoding or regulating the expression of tumor antigens, viral proteins, bacterial antigens, or any cellular gene product, the expression of which is to be down-regulated, may be used.

A host cell is contacted with an inhibitorily effective amount of the antisense inhibitor which diffuses into the cell, hybridizes to the sequence encoding the undesirable gene product and arrests its expression. An inhibitorily effective amount as used herein includes dosages of between about 0.1 ng to about 1 mg per kilogram per day. The antisense inhibitor may be contacted with cells in vitro or in vivo. When administered in vivo, the antisense inhibitor may be administered intravenously, or via another parenteral route. The inhibitor may be provided in an emulsion with lipids, including cationic lipids, encapsulated in liposomes, or in any number of other known pharmaceutically acceptable carriers. In vitro, the compounds of this invention are powerful tools for elucidating the effects of specifically turning-off expression of a targeted gene product. Specific primary sequences which may be used in the analog oligonucleotides of this invention and used as disclosed herein may include sequences complimentary to viral antigens, tumor antigens, or normal or abnormal cellular genes. Because the method of this invention is not limited to incorporation of only pyrimidines and also permits inclusion of adjacent purines, any primary sequence may be prepared. Suggested target

sequences are, for example, disclosed by Milligan et al., J. Med. Chem. 36 No.14:1923-1937, 1993, and the references cited therein.

According to the instant disclosure, any of the following compounds may be prepared according to the method of this invention and used as disclosed herein:

5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine;

N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-2'-deoxyadenosine;

5'-*O*-Dimethoxytrityl-3'-*O*-methylthiomethyl-thymidine;

5'-*S*-Acetyl-3'-*O*-methylthiomethyl-5'-deoxy-5'-thiothymidine;

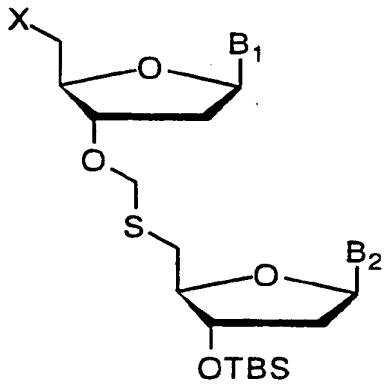
N^4-Benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-methylthiomethyl-2'-deoxycytidine;

3'-*O*-*t*-Butyldimethylsilyl-5'-deoxy-5'-thiothymidine;

N^4-Benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine;

N^6-Benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine;

N^2-Isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine;



5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=T, B_2=T, X=OTBS);

5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (B_1=T, B_2=C^{Bz}, X=OTBS);

5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=T, B_2=A^{Bz}, X=OTBS);

5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine (B_1=T, B_2=G^{iBu}, X=OTBS);

N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=A^{Bz}, B_2=T, X=OTBS);

N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (B_1=A^{Bz}, B_2=C^{Bz}, X=OTBS);

N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=A^{Bz}, B_2=A^{Bz}, X=OTBS);

N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine (B_1=A^{Bz}, B_2=G^{iBu}, X=OTBS);

5'-*O*-Dimethoxytrityl-3'-*O*-methylenethymidylyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=T, B_2=T, X=ODMT);

5'-*S*-Acetyl-3'-*O*-methylene-5'-deoxy-5'-thio-thymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=T, B_2=A^{Bz}, X=AcS);

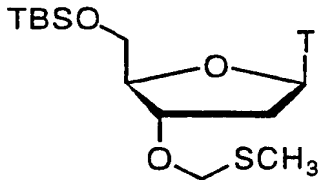
N^4-Benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-methylene-2'-deoxycytidylyl-(3'-5')-3'-*O*-methylene-5'-deoxy-5'-thiothymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine.

The following examples are provided to further describe how the invention may be put into practice, without limiting the invention to the specifics of these examples:

EXAMPLE 1

PREPARATION OF NUCLEOSIDE DONORS

Donor 1: 5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine



Step 1: 5'-*O*-*t*-Butyldimethylsilyl-thymidine

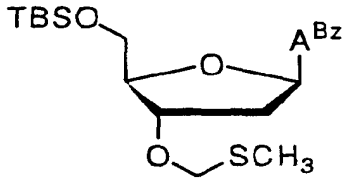
A solution of thymidine (6.1 g), imidazole (3.8 g) and *t*-butyldimethylsilyl chloride (4.3 g) in DMF (930 mL) was stirred at room temperature for 2 hours before EtOAc (150 mL) was added. The organic phase was washed with HCl 5%, water and brine, dried ($MgSO_4$) and evaporated. Flash chromatography of the residue (silica gel; hexane/EtOAc (80:20)) afforded the title compound as a white solid.

Step 2: 5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine

Sodium hydride (80% in mineral oil; 0.78 g) was added to a 0°C solution of the alcohol from Step 1 (2.00 g) in THF (66 mL). The mixture was stirred for 20 minutes at 0°C. Sodium iodide (0.92 g), HMPA (5.6 mL) and chloromethyl methyl sulfide (0.52 mL) were then consecutively added and the reaction mixture was stirred at room

temperature for 1.5 hour before water was added. The aqueous phase was extracted with EtOAc and the combined organic phases were dried ($MgSO_4$) and evaporated. Flash chromatography of the residue (silica gel; hexane/EtOAc (50:50)) afforded the title compound as a colorless gum.

Donor 2: N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-2'-deoxyadenosine



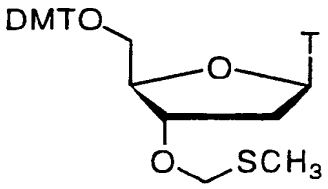
Step 1: N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-2'-deoxyadenosine

Following the procedure described for Donor 1 Step 1, but substituting N^6-benzoyl-2'-deoxyadenosine (*J. Am. Chem. Soc.* **1982**, *104*, 1316) for thymidine, the title compound was obtained as a white solid.

Step 2: N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-2'-deoxyadenosine

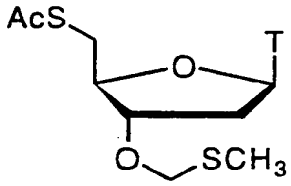
A solution of N^6-benzoyl-5'-*O*-*t*-butyldimethylsilyl-2'-deoxyadenosine from Step 1 (9.51 g), acetic acid (20 mL) and acetic anhydride (66 mL) in DMSO (100 mL) was stirred at room temperature for 17 hours before it was slowly poured in a 0°C solution of potassium carbonate (100 g) in water (1 L).The aqueous phase was extracted with chloroform (3 X 200 mL) and the combined organic phases were washed with water and brine, dried ($MgSO_4$) and evaporated. Flash chromatography of the residue (silica gel; hexane/EtOAc (30:70)) afforded the title compound as a white solid.

Donor 3: 5'-*O*-Dimethoxytrityl-3'-*O*-methylthiomethyl-thymidine



Following the procedure described for Donor 1 Step 2, but substituting 5'-*O*-dimethoxytritylthymidine (*J. Am. Chem. Soc.* **1963**, *85* , 3821) for 5'-*O*-*t*-butyldimethylsilyl-thymidine, the title compound was obtained as a white solid.

Donor 4: 5'-*S*-Acetyl-3'-*O*-methylthiomethyl-5'-deoxy-5'-thiothymidine



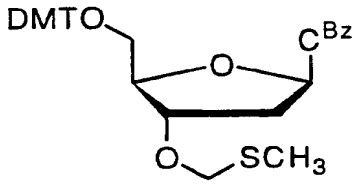
Step 1: 3'-*O*-methylthiomethyl-thymidine

p-Toluenesulfonic acid monohydrate (2.33 g) was added to a 0°C solution of 5'-*O*-dimethoxytrityl-3'-*O*-methylthiomethyl-thymidine (Donor 3, 3.13 g) in dichloromethane (35 mL) and methanol (15 mL). After being stirred at 0°C for 15 minutes, the reaction mixture was treated with 5% aqueous sodium bicarbonate and extracted with dichloromethane. The organic layer was dried ($MgSO_4$) and evaporated. Flash chromatography of the residue (silica gel; hexane/EtOAc (10:90)) afforded the title compound as a white solid.

Step 2: 5'-*S*-Acetyl-3'-*O*-methylthiomethyl-5'-deoxy-5'-thiothymidine

Diisopropyl azodicarboxylate (1.21 mL) was added to a 0°C solution of triphenylphosphine (1.61 g) in THF (15 mL). The mixture was stirred for 30 minutes at 0°C. 3'-*O*-methylthiomethyl-thymidine from Step 1 (0.93 g), THF (10 mL) and thiolacetic acid (0.44 mL) were consecutively added and the reaction mixture was stirred for 40 minutes at 0°C and 50 minutes at room temperature. The volatiles were then evaporated and flash chromatography of the residue (silica gel; hexane/EtOAc (40/60)) afforded the title compound as a yellow gum.

Donor 5: N^4-Benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-methylthiomethyl-2'-deoxycytidine

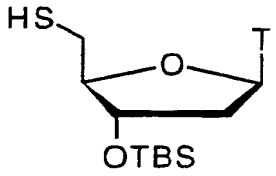


A mixture of N^4-benzoyl-5'-*O*-dimethoxytrityl-2'-deoxycytidine (*J. Am. Chem. Soc.* **1963**, *85* , 3821) (5.84 g), 2,6-lutidine (3.5 mL), dimethyl sulfide (18.4 mL) and benzoyl peroxide (24.2 g) in acetonitrile (130 mL) and dichloromethane (130 mL) was stirred at room temperature for 5 hours. Ethyl acetate was then added and the reaction mixture was washed with water, saturated aqueous ammonium chloride and brine. The organic layer was dried ($MgSO_4$) and evaporated. Flash chromatography of the residue (silica gel; hexane/EtOAc (25:75)) afforded the title compound as a white solid.

EXAMPLE 2

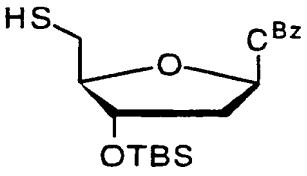
PREPARATION OF NUCLEOSIDE ACCEPTORS

Acceptor 1: 3'-*O*-*t*-Butyldimethylsilyl-5'-deoxy-5'-thiothymidine



This compound was prepared according to the procedure described in *Can. J. Chem.* **1992**, *70*, 1573.

Acceptor 2: N^4-Benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine



Step 1: 5'-*S*-Acetyl-N^4-benzoyl-2',5'-dideoxy-5'-thiocytidine

Following the procedure described for Donor 4 Step 2, but substituting N^4-benzoyl-2'-deoxycytidine (*J. Am. Chem. Soc.* **1982**, *104*, 1316) for 3'-*O*-methylthiomethyl-thymidine, the title compound was obtained as a white solid.

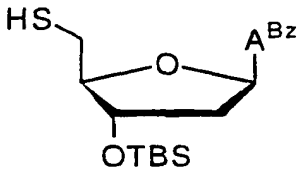
Step 2: 5'-*S*-Acetyl-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine

Following the procedure described for Donor 1 Step 1, but substituting 5'-*S*-acetyl-N^4-benzoyl-2',5'-dideoxy-5'-thiocytidine from Step 1 for thymidine, the title compound was obtained as a white solid.

Step 3: N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine

A methanolic sodium hydroxide solution (0.5 N; 7.0 mL) was added to a deoxygenated solution of 5'-*S*-acetyl-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (1.60 g) from Step 2 in methanol (20 mL).The reaction mixture was stirred at 0°C for 1 hour before it was neutralized by the addition of acidic Amberlyst 15 resin. The resin was filtered and washed thoroughly with methanol and the filtrate was evaporated. Flash chromatography of the residue (silica gel; hexane/EtOAc (50:50)) afforded the title compound as a white solid.

Acceptor 3: N^6-Benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine



Step 1: 5'-*S*-Acetyl-N^6-benzoyl-2',5'-dideoxy-5'-thioadenosine

Following the procedure described for Donor 4 Step 2, but substituting N^6-benzoyl-2'-deoxyadenosine (*J. Am. Chem. Soc.* **1982**, *104*, 1316) for 3'-*O*-methylthiomethyl-thymidine, the title compound was obtained as a white solid.

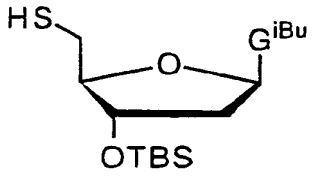
Step 2: 5'-*S*-Acetyl-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine

Following the procedure described for Donor 1 Step 1, but substituting 5'-*S*-acetyl-N^6-benzoyl-2',5'-dideoxy-5'-thioadenosine from Step 1 for thymidine, the title compound was obtained as a white solid.

Step 3: N^6-Benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine

Following the procedure described for Acceptor 2 Step 3, but substituting 5'-*S*-acetyl-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine from Step 2 for 5'-*S*-acetyl-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine, the title compound was obtained as a white solid.

Acceptor 4: N^2-Isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine



Step 1: 5'-*S*-Acetyl-N^2-isobutyryl-2',5'-dideoxy-5'-thioguanosine

Following the procedure described for Donor 4 Step 2, but substituting N^2-isobutyryl-2'-deoxyguanosine (*J. Am. Chem. Soc.* **1982**, *104*, 1316) for 3'-*O*-methylthiomethyl-thymidine, the title compound was obtained as a white solid.

Step 2: 5'-*S*-Acetyl-N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine

Following the procedure described for Donor 1 Step 1, but substituting 5'-*S*-acetyl-N^2-isobutyryl-2',5'-dideoxy-5'-thioguanosine from Step 1 for thymidine, the title compound was obtained as a white solid.

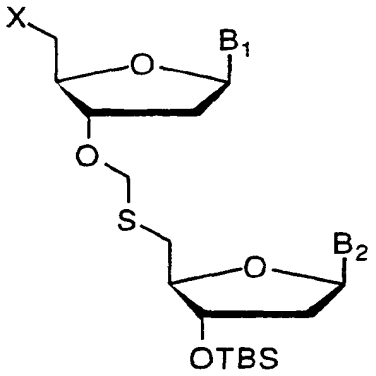
Step 3: N^2-Isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine

Following the procedure described for Acceptor 2 Step 3, but substituting 5'-*S*-acetyl-N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine from Step 2 for 5'-*S*-acetyl-N^4-benzoyl-3'-*O*-*t*-

butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine, the title compound was obtained as a white solid.

EXAMPLE 3

PREPARATION OF 5'-THIOFORMACETAL DINUCLEOTIDE ANALOGS



Dinucleotide Analog 1: 5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylene-thymidylyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=T, B_2=T, X=OTBS)

To a mixture of 5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthio-methyl-thymidine (Donor 1; 100 mg), *N*,*N*-diisopropyl-ethylamine (46 µL) and 3 Å molecular sieve (100 mg) in dichloromethane (1.5 mL) stirred at 0°C was added sulfuryl chloride (20 µL). After 1 minute, cyclohexene (38 µL) was added and the cold bath was removed. After 10 minutes at room temperature, a solution of 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (Acceptor 1; 91 mg) and *N*,*N*-diisopropylethyl-amine (46 µL) in dichloromethane (1.0 mL) was added to the reaction mixture. After 3.5 hours, the volatiles were evaporated and flash chromatography of the residue (silica gel; EtOAc) afforded the title compound as a pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.10 (s, 6H), 0.12 (s, 6H), 0.90 (s, 9H), 0.93 (s, 9H), 1.92 (d, J=0.9 Hz, 3H), 1.94 (s, 3H), 1.95 (m, 1H), 2.15 (m, 1H), 2.25 (m, 1H), 2.43 (dd, J=12.2, 5.5 Hz, 1H), 2.85 (dd, J=13.8, 5.9 Hz, 1H), 2.96 (dd, J=13.8, 4.9 Hz, 1H), 3.78 (dd, J=11.2, 2.5 Hz, 1H), 3.87 (dd, J=11.2, 3.0 Hz, 1H), 4.02 (m, 1H), 4.10 (bs, 1H), 4.29 (m, 1H), 4.45 (d, 1H), 4.72 (s, 2H), 6.20 (m, 1H), 6.27 (dd, J=8.6, 5.5 Hz, 1H), 7.27 (s, 1H), 7.46 (s, 1H), 9.08 (bs, 2H).

Dinucleotide Analog 2: 5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (B_1=T, B_2=C^{Bz}, X=OTBS)

Following the procedure described for Dinucleotide Analog 1, but substituting N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (Acceptor 2) for 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine, the title compound was obtained as a pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.09 (s, 6H), 0.13 (s, 6H), 0.90 (s, 9H), 0.93 (s, 9H), 1.92 (d, J=1.1 Hz, 3H), 2.04 (m, 1H), 2.15 (m, 1H), 2.43 (m, 1H), 2.61 (m, 1H), 2.88 (dd, J=13.7, 6.1 Hz, 1H), 2.97 (dd, J=13.7, 4.9 Hz, 1H), 3.80 (dd, J=11.3, 2.6 Hz, 1H), 3.88 (dd, J=11.3, 3.1 Hz, 1H), 4.14 (m, 2H), 4.24 (m, 1H), 4.44 (d, 1H), 4.75 (ABq, J=11.6 Hz, 2H), 6.22 (t, 1H), 6.28 (dd, J=8.6, 5.5 Hz, 1H), 7.46 (d, J=1.2 Hz, 1H), 7.52 (m, 2H), 7.62 (m, 2H), 7.91 (m, 2H), 8.12 (d, J=7.3 Hz, 1H), 8.35 (b, 1H), 8.90 (b, 1H).

Dinucleotide Analog 3: 5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=T, B_2=A^{Bz}, X=OTBS)

Following the procedure described for Dinucleotide Analog 1, but substituting N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (Acceptor 3) for 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine, the title compound was obtained as an amber gum.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.10 (s, 6H), 0.14 (s, 3H), 0.15 (s, 3H), 0.91 (s, 9H), 0.93 (s, 9H), 1.88 (d, J=1.0 Hz, 3H), 1.95 (m, 1H), 2.47 (m, 1H), 2.89-3.08 (m, 4H), 3.74 (dd, J=11.3, 2.5 Hz, 1H), 3.83 (dd, J=11.3, 3.0 Hz, 1H), 4.03 (m, 1H), 4.18 (m, 1H), 4.38 (d, J=6.0 Hz, 1H), 4.62-4.69 (m, 3H), 6.22 (dd, J=8.6, 5.5 Hz, 1H), 6.45 (t, 1H), 7.44 (d, J=1.2 Hz, 1H), 7.49 (m, 2H), 7.56 (m, 1H), 8.05 (m, 2H), 8.29 (s, 1H), 8.81 (s, 1H), 9.63 (bs, 1H).

Dinucleotide Analog 4: 5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine (B_1=T, B_2=G^{iBu}, X=OTBS)

Following the procedure described for Dinucleotide Analog 1, but substituting N^2-Isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine (Acceptor 4) for 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine, the title compound was obtained as an amber gum.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.11 (s, 6H), 0.12 (s, 6H), 0.90 (s, 9H), 0.92 (s, 9H), 1.25 (d, 3H), 1.26 (d, 3H), 1.92 (s, 3H), 1.97 (m, 1H), 2.39 (m, 2H), 2.68 (m, 2H), 2.83 (dd, J=14.1, 5.9 Hz, 1H), 2.94 (dd, J=14.0, 5.2 Hz, 1H), 3.76 (dd, J=11.2, 2.2 Hz, 1H), 3.84 (dd, J=11.2, 2.6 Hz, 1H), 4.07 (m, 1H), 4.10 (m, 1H), 4.35 (m, 1H), 4.46 (dd, J=9.9, 4.6 Hz, 1H), 4.62 (s, 2H), 6.24 (m, 2H), 7.48 (s, 1H), 7.87 (s, 1H), 9.10 (bs, 2H).

Dinucleotide Analog 5: N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=A^{Bz}, B_2=T, X=OTBS)

Following the procedure described for Dinucleotide Analog 1, but substituting N^6-benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-2'-deoxyadenosine (Donor 2) for 5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine, the title compound was obtained as a pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.10 (s, 6H), 0.11 (s, 6H), 0.90 (s, 9H), 0.91 (s, 9H), 1.94 (d, J=0.7 Hz, 3H), 2.18 (m, 1H), 2.30 (m, 1H), 2.64 (m, 1H), 2.74 (m, 1H), 2.88 (dd, J=13.9, 5.8 Hz, 1H), 2.99 (dd, J=13.9, 5.0 Hz, 1H), 3.81 (dd, J=11.0, 3.4 Hz, 1H), 3.87 (dd, J=10.9, 4.8 Hz, 1H), 4.05 (dd, J=10.0, 5.2 Hz, 1H), 4.21 (bs, 1H), 4.31 (m, 1H), 4.65 (m, 1H), 4.78 (ABq, J=11.5 Hz, 2H), 6.20 (t, 1H), 6.49 (dd, J=7.6, 5.8 Hz, 1H), 7.27 (d, J=0.9 Hz, 1H), 7.53 (m, 2H), 7.61 (m, 1H), 8.03 (m, 2H), 8.31 (s, 1H), 8.35 (bs, 1H), 8.81 (s, 1H), 9.03 (bs, 1H).

Dinucleotide Analog 6: N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (B_1=A^{Bz}, B_2=C^{Bz}, X=OTBS)

Following the procedure described for Dinucleotide Analog 1, but substituting N^6-benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-2'-deoxyadenosine (Donor 2) for 5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine and substituting N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (Acceptor 2) for 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine, the title compound was obtained as a pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.10 (s, 6H), 0.11 (s, 6H), 0.90 (s, 9H), 0.92 (s, 9H), 2.16 (m, 1H), 2.63 (m, 1H), 2.82 (m, 1H), 2.91 (dd, J=13.8, 6.2 Hz, 1H), 2.99 (dd, J=13.8, 4.8 Hz, 1H), 3.82 (dd, J=11.0, 3.5 Hz, 1H), 3.89 (dd, J=10.9, 4.9 Hz, 1H), 4.17 (dd, J=10.8, 4.8 Hz, 1H), 4.24 (m, 3H), 4.68 (m, 1H), 4.82 (ABq, J=11.6 Hz, 2H), 6.23 (t, 1H), 6.52 (dd, J=7.7, 5.9 Hz, 1H), 7.53 (m, 5H), 7.61 (m, 2H), 7.88 (m, 2H), 8.02 (m, 2H), 8.14 (m, 1H), 8.32 (s, 1H), 8.66 (bs, 1H), 8.79 (s, 1H), 8.96 (bs, 1H).

Dinucleotide Analog 7: N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=A^{Bz}, B_2=A^{Bz}, X=OTBS)

Following the procedure described for Dinucleotide Analog 1, but substituting N^6-benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-2'-deoxyadenosine (Donor 2) for 5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine and substituting N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (Acceptor 3) for 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine, the title compound was obtained as a pale yellow solid.

^{1}H NMR (400 MHz, CDCl3) δ 0.08 (s, 3H), 0.09 (s, 3H), 0.14 (s, 3H), 0.15 (s, 3H), 0.90 (s, 9H), 0.94 (s, 9H), 2.49 (m, 1H), 2.59 (m, 1H), 2.71 (m, 1H), 2.98 (m, 3H), 3.77 (dd, J=11.0, 3.4 Hz, 1H), 3.85 (dd, J=11.0, 4.8 Hz, 1H), 4.15 (m, 1H), 4.20 (m, 1H), 4.62 (m, 2H), 4.70 (ABq, J=11.6 Hz, 2H), 6.46 (m, 2H), 7.52 (m, 4H), 7.60 (m, 2H), 8.02 (m, 4H), 8.23 (s, 1H), 8.30 (s, 1H), 8.76 (s, 1H), 8.79 (s, 1H), 9.01 (bs, 1H), 9.07 (bs, 1H).

Dinucleotide Analog 8: N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine (B_1=A^{Bz}, B_2=G^{iBu}, X=OTBS)

Following the procedure described for Dinucleotide Analog 1, but substituting N^6-benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-2'-deoxyadenosine (Donor 2) for 5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine and substituting N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguano-sine (Acceptor 4) for 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine, the title compound was obtained as a pale yellow solid.

^{1}H NMR (400 MHz, CDCl3) δ 0.08 (s, 6H), 0.12 (s, 3H), 0.13 (s, 3H), 0.89 (s, 9H), 0.92 (s, 9H), 1.24 (s, 3H), 1.26 (s, 3H), 2.40 (m, 1H), 2.60-2.75 (m, 4H), 2.87 (dd, J=14.0, 6.0 Hz, 1H), 2.96 (dd, J=14.0, 5.3 Hz, 1H), 3.78 (dd, J=10.9, 3.5 Hz, 1H), 3.84 (dd, J=10.9, 4.8 Hz, 1H), 4.15 (m, 2H), 4.48 (dd, J=10.5, 4.5 Hz, 1H), 4.60 (m, 1H), 4.71 (ABq, J=11.6 Hz, 2H), 6.20 (t, 1H), 6.46 (dd, J=7.6, 6.1 Hz, 1H), 7.51

(m, 2H), 7.60 (m, 1H), 7.87 (s, 1H), 8.01 (m, 2H), 8.30 (s, 1H), 8.72 (bs, 1H), 8.79 (s, 1H), 9.07 (bs, 1H).

Dinucleotide Analog 9: 5'-*O*-Dimethoxytrityl-3'-*O*-methylenethymidylyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=T, B_2=T, X=ODMT)

Following the procedure described for Dinucleotide Analog 1, but substituting 5'-*O*-dimethoxytrityl-3'-*O*-methylthiomethyl-thymidine (Donor 3) for 5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine, the title compound was obtained as a pale yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.08 (s, 6H), 0.89 (s, 9H), 1.49 (d, J=1.0 Hz, 3H), 1.90 (d, J=1.0 Hz, 3H), 2.16 (m, 1H), 2.27 (m, 2H), 2.48 (m, 1H), 2.76 (dd, J=14.0, 6.0 Hz, 1H), 2.89 (dd, J=13.9, 4.8 Hz, 1H), 3.32 (dd, J=10.5, 2.8 Hz, 1H), 3.47 (dd, J=10.5, 3.2 Hz, 1H), 3.79 (s, 6H), 3.98 (dd, J=10.5, 4.8 Hz, 1H), 4.12 (dd, J=5.0, 2.3 Hz, 1H), 4.26 (m, 1H), 4.62 (m, 1H), 4.67 (ABq, J=11.6 Hz, 2H), 6.19 (t, 1H), 6.34 (dd, J=8.1, 5.7 Hz, 1H), 6.84 (d, 4H), 7.22-7.32 (m, 8H), 7.40 (m, 2H), 7.57 (d, J=1.2 Hz, 1H), 8.96 (s, 2H).

Dinucleotide Analog 10: 5'-*S*-Acetyl-3'-*O*-methylene-5'-deoxy-5'-thiothymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=T, B_2=A^{Bz}, X=AcS)

Following the procedure described for Dinucleotide Analog 1, but substituting 5'-*S*-acetyl-3'-*O*-methylthiomethyl-5'-deoxy-5'-thiothymidine (Donor 4) for 5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine and substituting N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (Acceptor 3) for 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine, the title compound was obtained as a beige solid.

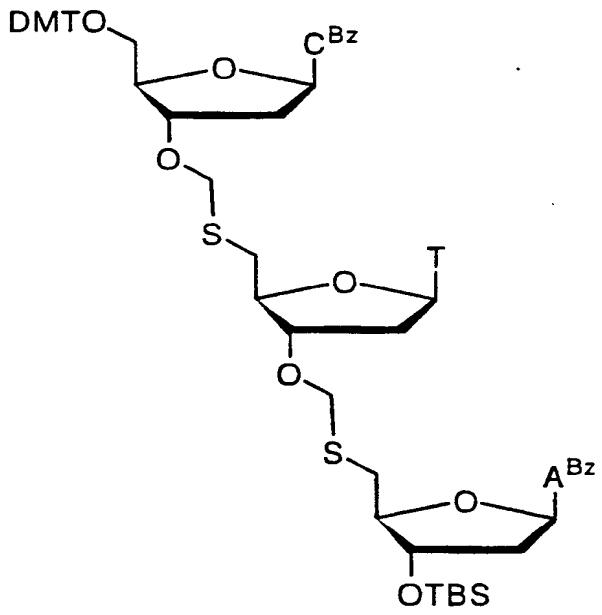
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.14 (s, 3H), 0.15 (s, 3H), 0.93 (s, 9H), 1.91 (d, J=1.1 Hz, 3H), 2.05 (m, 1H), 2.35 (m, 1H), 2.37 (s,

3H), 2.48 (m, 1H), 2.91-3.02 (m, 3H), 3.18 (m, 2H), 4.03 (m, 1H), 4.17 (m, 2H), 4.64 (m, 3H), 6.10 (dd, J=8.0, 5.9 Hz, 1H), 6.45 (t, 1H), 7.18 (d, J=1.2 Hz, 1H), 7.49 (m, 2H), 7.56 (m, 1H), 8.05 (m, 2H), 8.31 (s, 1H), 8.81 (s, 1H), 9.63 (bs, 1H).

EXAMPLE 4

PREPARATION OF A 5'-THIOFORMACETAL TRINUCLEOTIDE ANALOG

Trinucleotide Analog 1: N^4-Benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-methylene-2'-deoxycytidylyl-(3'-5')-3'-*O*-methylene-5'-deoxy-5'-thiothymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine



Step 1: 3'-*O*-Methylene-5'-deoxy-5'-thiothymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine

Following the procedure described for Acceptor 2 Step 3, but substituting 5'-*S*-acetyl-3'-*O*-methylene-5'-deoxy-5'-thio-thymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (Dinucleotide Analog 10) for 5'-*S*-acetyl-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine, the title compound was obtained as a yellow solid.

Step 2: N^4-Benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-methylene-2'-deoxycytidylyl-(3'-5')-3'-*O*-methylene-5'-deoxy-5'-thio-thymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine

Following the procedure described for Dinucleotide Analog 1, but substituting N^4-benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-methylthiomethyl-2'-deoxycytidine (Donor 5) for 5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine and substituting 3'-*O*-Methylene-5'-deoxy-5'-thiothymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine from Step 1 for 3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine, the title compound was obtained as a beige solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.13 (s, 6H), 0.92 (s, 9H), 1.87 (s, 3H), 2.09 (m, 1H), 2.22 (m, 1H), 2.35 (m, 1H), 2.50 (m, 1H), 2.77 (m, 3H), 2.97 (m, 3H), 3.39 (m, 1H), 3.47 (m, 1H), 3.78 (s, 6H), 4.08 (m, 1H), 4.16 (m, 1H), 4.19 (m, 1H), 4.24 (m, 1H), 4.51 (m, 1H), 4.64 (m, 5H), 6.09 (t, 1H), 6.21 (t, 1H), 6.43 (t, 1H), 6.85 (d, 4H), 7.20-7.60 (m, 19H), 7.88 (m, 2H), 8.05 (m, 2H), 8.22 (d, 1H), 8.28 (s, 1H), 8.70 (bs, 1H), 8.78 (s, 1H), 9.29 (bs, 1H), 9.45 (bs, 1H).

EXAMPLE 5

EXAMPLES OF SPECIFIC SEQUENCES OF ANTISENSE INHIBITORS AND THEIR APPLICATION TO INHIBITION OF GENE EXPRESSION

Methods of synthesizing and using antisense inhibitors were recently reviewed by Milligan et al., [J. Med. Chem. 36 No.14:1923-1937, 1993]. References cited therein as to appropriate targets and primary sequences for antisense inhibitors are all amenable to use according to the method of the instant invention which enables production of antisense inhibitors incorporating essentially any known sequence of purines and pyrimidines. See also, for example WO8301451; WO9401550; WO9405268; US Patent 5,302,706; 5,298,612; 5,294,698; 5,294,533; 5,292,875; 5,284,755; 5,279,957; 5,276,017; 5,273,656; 5,272,065; 5,264,564; 5,264,618; 5,271,941; 5,272,250; 5,264,563; 5,262,522; 5,256,648; 5,252,723 all of which are herein incorporated by reference for these purposes.

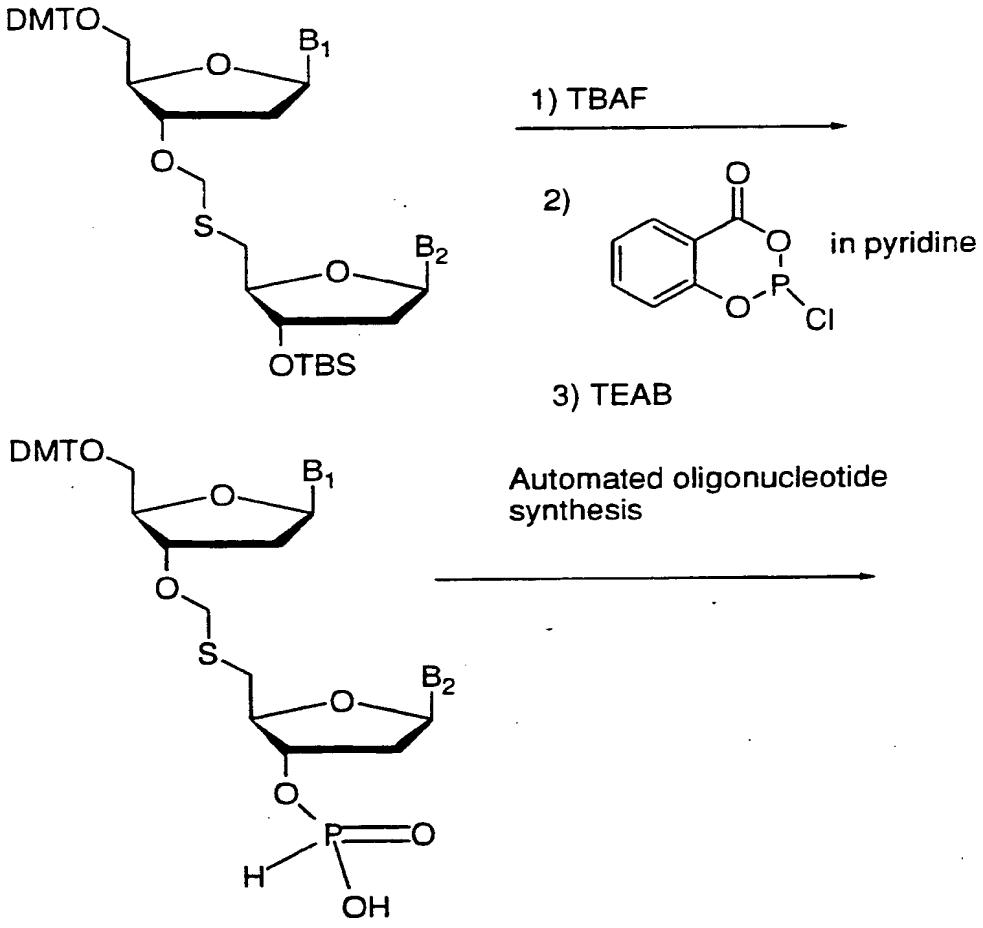
EXAMPLE 6

INCORPORATION OF 5'-THIOFORMACETAL LINKED OLIGODEOXYNUCLEOTIDES INTO PHOSPHODIESTER LINKED NATURAL OLIGODEOXYNUCLEOTIDE SEQUENCES

Dinucleotide and longer analogs of this invention are incorporated into longer sequences of phosphodiester linked natural oligonucleotides as follows:

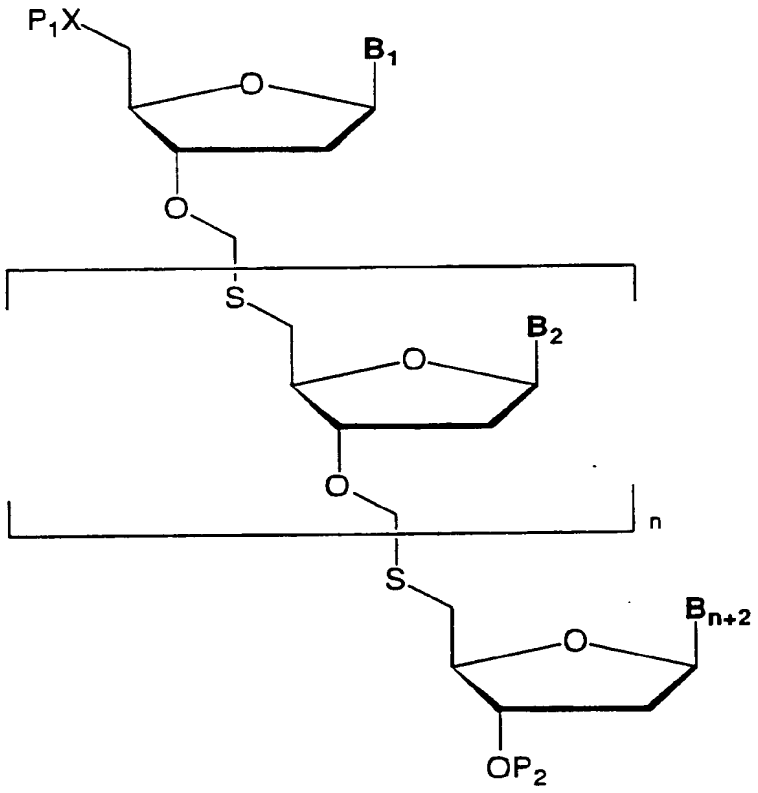
A 3'-end silylated di- or oligonucleotide analog of this invention is treated with tetrabutylammonium fluoride to afford a 3'-unprotected analog. This compound is converted into an H-phosphonate by the method of Marugg et al., [Tetrahedron Lett. 27:2661, 1986]. This derivative is then used in solid-phase automated oligodeoxynucleotide synthesis using H-phosphonate chemistry which is well known in the art

[see for example Froehler et al., Nuc. Acids Res. 14:5399, 1986]. This chemistry is summarized in the following scheme:



WHAT IS CLAIMED IS:

1. A compound comprising an oligonucleotide analog of formula:

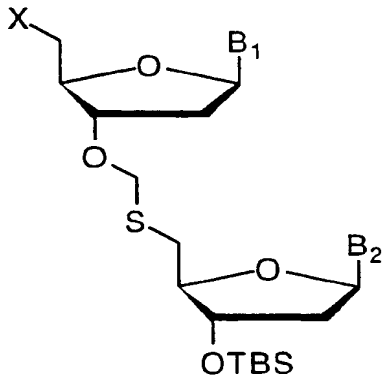


wherein:
B_1, B_2, and B_{n+2} are naturally occurring or non-naturally occurring purine or pyrimidine nucleic acid bases;
P_1 and P_2 are independently H, lower alkyl, acyl, substituted or unsubstituted trityl or trialkylsilyl;
X is O, or S; and
n is an number from 0 to 28.

2. The compound of Claim 1 wherein B_1, B_2, and B_{n+1} are selected from adenine, thymine, guanine, cytosine, uracil, and inosine.

3. The compound:
5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylthiomethyl-thymidine;
N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylthiomethyl-2'-deoxyadenosine;
5'-*O*-Dimethoxytrityl-3'-*O*-methylthiomethyl-thymidine;
5'-*S*-Acetyl-3'-*O*-methylthiomethyl-5'-deoxy-5'-thiothymidine;
N^4-Benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-methylthiomethyl-2'-deoxycytidine;
3'-*O*-*t*-Butyldimethylsilyl-5'-deoxy-5'-thiothymidine;
N^4-Benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine;
N^6-Benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine; or
N^2-Isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine.

4. The compound:



5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=T, B_2=T, X=OTBS);

5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (B_1=T, B_2=C^{Bz}, X=OTBS);

5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=T, B_2=A^{Bz}, X=OTBS);

5'-*O*-*t*-Butyldimethylsilyl-3'-*O*-methylenethymidylyl-(3'-5')-N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine (B_1=T, B_2=G^{iBu}, X=OTBS);

N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=A^{Bz}, B_2=T, X=OTBS);

N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^4-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thiocytidine (B_1=A^{Bz}, B_2=C^{Bz}, X=OTBS);

N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=A^{Bz}, B_2=A^{Bz}, X=OTBS);

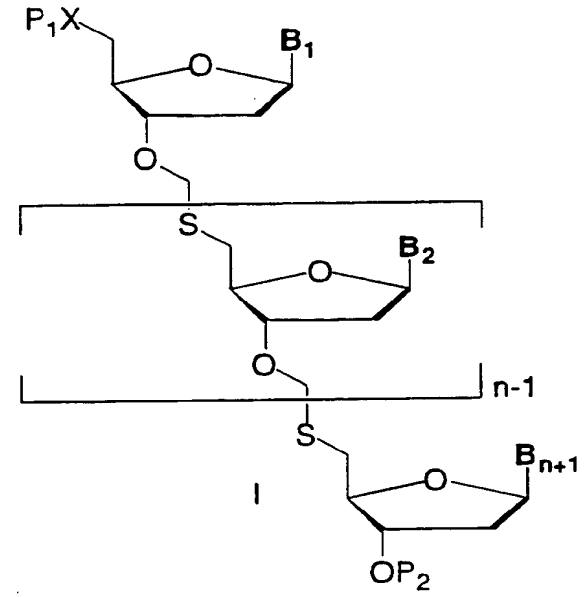
N^6-Benzoyl-5'-*O*-*t*-butyldimethylsilyl-3'-*O*-methylene-2'-deoxyadenosynyl-(3'-5')-N^2-isobutyryl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioguanosine (B_1=A^{Bz}, B_2=G^{iBu}, X=OTBS);

5'-*O*-Dimethoxytrityl-3'-*O*-methylenethymidylyl-(3'-5')-3'-*O*-*t*-butyldimethylsilyl-5'-deoxy-5'-thiothymidine (B_1=T, B_2=T, X=ODMT); or

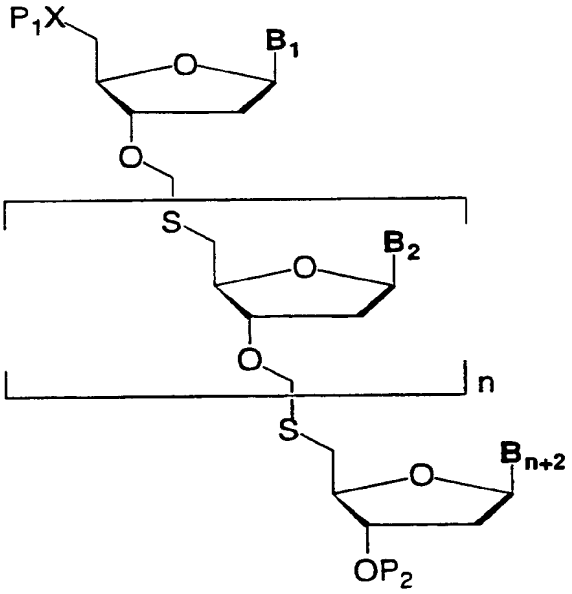
5'-*S*-Acetyl-3'-*O*-methylene-5'-deoxy-5'-thio-thymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine (B_1=T, B_2=A^{Bz}, X=AcS).

5. The compound:
N^4-Benzoyl-5'-*O*-dimethoxytrityl-3'-*O*-methylene-2'-deoxycytidylyl-(3'-5')-3'-*O*-methylene-5'-deoxy-5'-thiothymidylyl-(3'-5')-N^6-benzoyl-3'-*O*-*t*-butyldimethylsilyl-2',5'-dideoxy-5'-thioadenosine.

6. A method for making a compound of formula II starting with a compound of formula I:



I

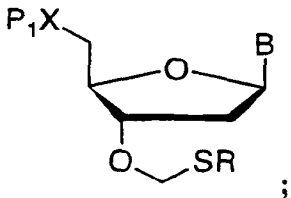


II

wherein:
B1, B2, Bn-1, Bn+1 and Bn+2 are naturally occurring or non-naturally occurring nucleic acid bases;

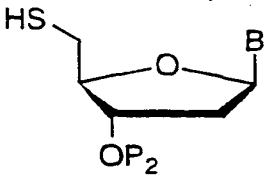
P_1 and P_2 , together are an oligomer up to a length of n, or are independently H, lower alkyl, acyl, substituted or unsubstituted trityl or trialkylsilyl;
X is O, or S; and
n is an number from 0 to 28;
which comprises:

a) Preparing a nucleoside donor of formula:



;

b) Preparing a nucleoside acceptor of formula:



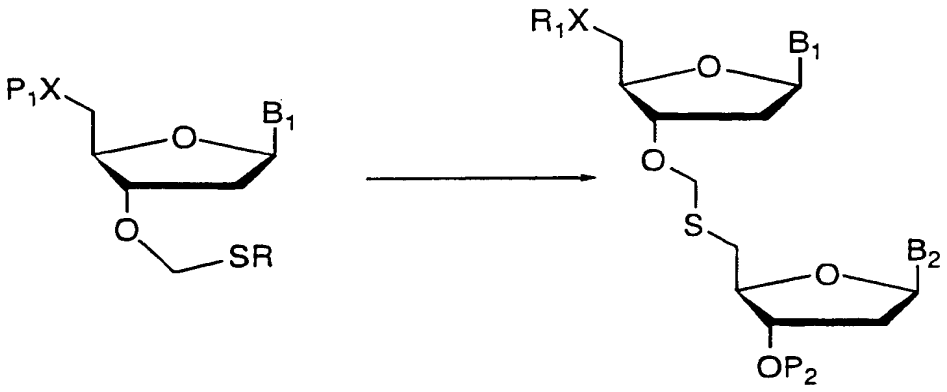
; and

c) Coupling the nucleoside donor and acceptor;

wherein:
B is a naturally occurring or non-naturally occurring nucleic acid purine or a pyrimidine base;
R is a lower alkyl;

d) Repeating steps (a)-(c) as many times as required to achieve the compound with the desired sequence having a total of n+2 bases.

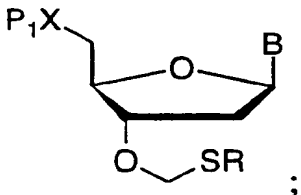
7. A method of making a compound of formula II starting with a compound of formula I:



I II

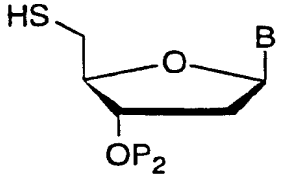
wherein:
B_1, and B_2 are naturally occurring or non-naturally occurring nucleic acid purine and pyrimidine bases;
P_1 and P_2 are independently H, lower alkyl, acyl, substituted or unsubstituted trityl, or trialkylsilyl; and
X is O, or S;
which comprises:

a) Preparing a nucleoside donor of formula:



;

b) Preparing a nucleoside acceptor of formula:



; and

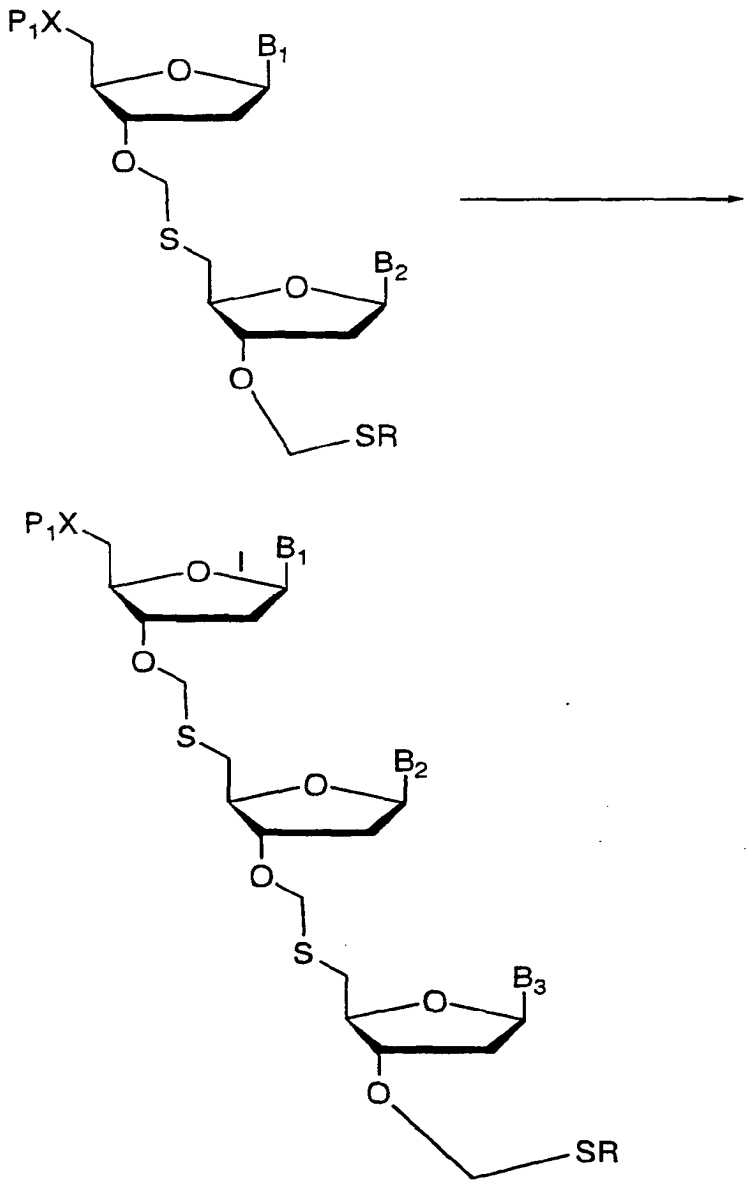
c) Coupling the nucleoside donor and acceptor;

wherein:

B is a naturally occurring or non-naturally occurring nucleic acid purine or a pyrimidine base;

R is a lower alkyl.

8. A method of making a compound of formula II starting with a compound of formula I:



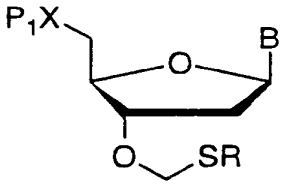
II

wherein:

B_1, B_2, and B_3 are naturally occurring or non-naturally occurring nucleic acid purine and pyrimidine bases;

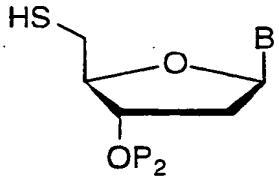
P_1 and P_2 are independently H, lower alkyl, acyl, substituted or unsubstituted trityl, trialkylsilyl or a dinucleotide analog with the priviso that only one of P_1 and P_2 is a dinucleotide; and
X is O, or S;
which comprises:

a) Preparing a nucleoside donor of formula:



;

b) Preparing a nucleoside acceptor of formula:



; and

c) Coupling the nucleoside donor and acceptor;

wherein:
B is a naturally occurring or non-naturally occurring nucleic acid purine or a pyrimidine base;
R is a lower alkyl.

9. The method of any one of Claims 6, 7, or 8 wherein step (a) comprises:
preparing a nucleoside donor with selective 5'-O-silylation of suitably base-protected deoxynucleosides, alkylating the secondary 3'-hydroxyl group with chloromethyl sulfide or by a Pummerer reaction to afford a 3'-O-methylthiomethylacetal donor.

10. The method of any one of Claim 6, 7, or 8 wherein step (b) comprises:
synthesizing a Mitsunobu reaction on the primary alcohol of suitably base-protected deoxynucleosides to afford 5'-S-acetyl nucleoside

derivatives. Silylation of the secondary alcohol followed by methanolysis of the thioester provides the desired 5'-thionucleoside derivatives used as nucleoside acceptors.

11. The method of any one of Claims 6, 7, or 8 wherein step (c) comprises:

i) mixing the nucleoside donor, N,N-diisopropylamine (about 1.4 equivalents) or a similar reagent and 3 angstrom molecular sieve in dichloromethane or a similar reagent and stirring at about 0°C;

ii) adding sulfuryl chloride (about 1.3 equivalents);

iii) adding cyclohexene (about 2 equivalents) to trap the methylsulfenyl chloride formed in situ and stirring for a short period of about 10 minutes at ambient temperature;

iv) adding a solution of the nucleoside acceptor (about 1.3 equivalents) and N,N-diisopropylamine (about 1.4 equivalents) or a similar reagent in dichloromethane or a similar solvent, and allowing the reaction to proceed for several hours;

v) removing the volatiles and fractionating the residue;

vi) recovering the 5'-thioformacetal linked oligonucleotide analog.

12. A method of inhibiting expression of undesirable gene sequences in a host cell which comprises contacting said cell with an inhibitorily effective amount of a compound of Claim 1, 2, 3, 4 or 5.

13. A pharmaceutical composition comprising a compound of Claim 1, 2, 3, 4 or 5 and a pharmaceutically acceptable carrier.

14. A gene expression antisense inhibitor pharmaceutical composition comprising an acceptable gene expression antisense inhibiting amount of a compound of Claim 1, 2, 3, 4 or 5, in association with a pharmaceutically acceptable carrier.

15. An antiviral pharmaceutical composition comprising an acceptable antivirally effective amount of a compound of Claim 1, 2, 3, 4 or 5, in association with a pharmaceutically acceptable carrier.

16. An anticancer pharmaceutical composition comprising an acceptable anticancerally effective amount of a compound of Claim 1, 2, 3, 4 or 5, in association with a pharmaceutically acceptable carrier.

17. Use of a compound of Claim 1, 2, 3, 4 or 5 in the manufacture of a medicament for antisense inhibition of gene expression, treatment or prevention of cancer.

18. Use of a compound of Claim 1, 2, 3, 4 or 5 as a gene expression antisense inhibitor, antiviral agent or anticancer agent.

19. A compound of Claim 1, 2, 3, 4 or 5 for use in the antisense inhibition of gene expression, treatment or prevention of viral infections or treatment or prevention of cancer.

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Filed on 13 May 1994 (13.05.94)

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(74) Agent: MURPHY, Kevin, P.; Swabey, Ogilvy, Renault, Suite 1600, 1981 McGill College, Montreal, Quebec H3A 2Y3 (CA).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

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(54) Title: ANTISENSE INHIBITORS OF GENE EXPRESSION

(57) Abstract

This invention is a new synthetic method for the preparation of oligonucleotide analogs containing a neutral 5'-thioformacetal internucleoside linkage and new di- and trinucleotide analogues containing purines and pyrimidines with neutral 5'-thioformacetal internucleoside linkages.

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GA	Gabon				

INTERNATIONAL SEARCH REPORT

Inte[redacted]l Application No
PCT/CA 95/00280

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/02 A61K31/70 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. AM. CHEM. SOC., vol.113, 19 October 0 pages 7767 - 8 M. MATTEUCCI ET AL. 'Deoxyoligonucleotides bearing neutral analogues of phosphodiester linkages recognize duplex DNA via triple-helix formation' cited in the application see the whole document --- -/--	1,2,4-19

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
14 September 1995	23 -01- 1996

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Bardili, W

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. ORG. CHEM., vol.58, pages 2983 - 91 R.J. JONES ET AL 'Synthesis and binding properties of pyrimidine oligodeoxynucleotide analogues containing neutral phosphodiester replacements: The formacetal and 3'-thioformacetal internucleoside linkages' cited in the application see the whole document	1,2,4-19
A	TETRAHEDRON LETT., vol.34, pages 6189 - 92 T. SUDHAKAR RAO ET AL. 'Synthesis of triple helix forming oligonucleotides with a stretched phosphodiester backbone' see the whole document	1,2,4-19
A	J. ORG. CHEM., vol.56, 19 October 0 pages 3869 - 82 Z. HUANG ET AL. 'Building blocks for oligonucleotide analogues with dimethylene sulfide, sulfoxide, and sulfone groups replacing phosphodiester linkages' see the whole document	1,2,4-19
A	TETRAHEDR. LETT., vol.32, 19 October 0 pages 7593 - 6 S. ZAVGORODNY ET AL. '1-Alkylthioalkylation of nucleoside hydroxyl functions and its synthetic applications' cited in the application see the whole document	6-11
A	ACTA CHEM. SCAND. SER. B, vol.37, pages 93 - 6 T. BENNECHE ET AL. 'Synthesis of alpha-haloalkyl aryl ethers from O,S-acetals' cited in the application	6-11

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INTERNATIONAL SEARCH REPORT

Inte[redacted]pplication No.
PCT/CA95/00280

Box I Observations where certain claims were found unsearchable (Continuation of item 1 f first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims : 1, 2, 4 - 19
2. Claim : 3

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 2, 4-19

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.